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(54) **Expression of viral proteins in plants**

Expression viraler Proteine in Pflanzen

Expression de protéines virales dans des plantes

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- MACLEAN J ET AL: "Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization", JOURNAL OF GENERAL VIROLOGY, SOCIETY FOR GENERAL MICROBIOLOGY, SPENCERS WOOD, GB, vol. 88, no. Part 5, 1 May 2007 (2007-05-01), pages 1460-1469, XP002490006, ISSN: 0022-1317, DOI: DOI:10.1099/VIR.0.82718-0

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Description

BACKGROUND TO THE INVENTION

[0001] This invention relates to the expression of protein in plants. In particular, this invention relates to a method of transgenic protein expression and intracellular localisation for high yield protein production. This invention also relates to the production of human papillomavirus (HPV) protein in a plant. This invention further relates to a rapid mechanism for evaluating protein expression within plants.

[0002] The use of transgenic plants for the large-scale production of heterologous proteins is gradually gaining widespread acceptance, and could be a platform for the cost-effective production of vaccine proteins. The generation of transgenic plants to investigate the expression characteristics of a large assortment of proteins and/or expression vectors is not always viable due to the time-consuming nature of this process. In contrast, transient expression systems are capable of rapidly evaluating protein expression of a variety of different proteins in a variety of plant species.

[0003] Another problem relating to the use of transgenic plants for large-scale production of proteins is the generally disappointingly low levels of heterologous protein produced. Plant plastids, especially the chloroplast, have successfully been transformed with recombinant vectors expressing heterologous protein to significantly increase the levels of protein produced per total soluble protein in the plant (see, for example, WO2005/011367 and WO2004/005467A2). Plastids are also able to import a variety of molecules, including proteins. There are different processes through which this occurs, one of which involves interaction with the thylakoid membrane of the plastid. Targeting of heterologous proteins to the plastid is a mechanism of significantly increasing protein accumulation within the plant cell.

[0004] *Agrobacterium*-mediated gene transfer has been utilised for many years to generate stably transformed plants. This method involves the transfer of the T-complex (a complex formed with the agrobacterial T-DNA and virulence gene products) from *Agrobacterium* strains to plant cells (Zupan *et al*., 2000). Any DNA located between the 25bp direct repeats (left and right borders) that delimit the single-stranded T-DNA is transferred into the plant cell nucleus (Zupan *et al*., 2000), where it integrates into the plant chromosome via illegitimate recombination (Somers and Makarevitch, 2004). Many of the T-DNA copies present in the nucleus do not integrate, but get transcribed, causing transient expression. Transient expression is not affected by the position effect, and can produce dramatically higher foreign protein levels than stable transformation (Kapila *et al*., 1997).

[0005] Two *Agrobacterium* infiltration (agroinfiltation) methods are commonly utilised: Injection and vacuum infiltration. *Agrobacterium* injection involves direct injection into the abaxial airspaces of a leaf, while the leaf is still attached to the plant (Voinnet *et al*., 2003). During vacuum infiltration a vacuum is applied to an agrobacterial suspension in which leaves are submerged (Kapila *et al.,* 1997). Although usually performed on just a few leaves, this technique can be scaled up: Researchers at Medicago Inc. (Quebec, Canada) are able to agroinfiltrate up to 7500 alfalfa leaves per week, and Stefan Schillberg and colleagues (Institute for Molecular Biotechnology, RWTH Aachen, Germany) have agroinfiltrated up to 100kg of tobacco leaves (Twyman 2004).

[0006] *Agrobacterium*-mediated transient expression peaks 60 to 72 hours post infiltration, and then declines sharply as a result of post-transcriptional gene silencing (PTGS) (Voinnet *et al*., 2003). PTGS or RNA interference is an adaptive antiviral defence response that limits virus replication and spread in plants. This process involves recognition of a target RNA and initiation of a sequence-specific RNA degradation pathway in the cytoplasm (Voinnet, 2001). Certain plant viruses encode silencing suppressors that have the ability to inhibit PTGS. Some of these silencing suppressors proteins, such as NSs of the tomato spotted wilt virus (TSWV) (Takeda *et al.,* 2002), and p19 of tomato bushy stunt virus (Voinnet *et al*., 2003) have been utilised to prolong and amplify *Agrobacterium*-mediated transient expression by the co-infiltration of *Agrobacterium* containing a silencing suppressor gene.

[0007] A need exists to optimise the expression of commercially viable proteins in plants to levels that would make plants a viable platform. In particular, a need exists for a method of producing HPV protein in a plant.

[0008] Further needs exist for vectors, transgenic plants or parts thereof and the progeny of such plants to effect the need as hereinbefore described.

SUMMARY OF THE INVENTION

[0009] According to a first aspect to the present invention there is provided a method of producing HPV L1 polypeptides in a plant comprising the steps of:

1. cloning a HPV L1 gene or nucleic acid encoding a functional equivalent into a vector adapted to target a polypeptide expressed in the cytoplasm to the chloroplasts present in the plant;
2. infiltrating at least a portion of the plant with the vector or transforming plant tissue with the vector so as to transiently express the HPV L1 polypeptides in the cytoplasm and then import the HPV L1 polypeptides into the chloroplasts and/or to create a transgenic plant wherein the HPV L1 polypeptides are expressed in the cytoplasm

and then imported into the chloroplasts; and

3. recovering the HPV L1 polypeptides expressed by the plant.

[0010] The term "plant" is intended to encompass all living organisms which lack specialised sense organs and the power of voluntary movement. As such, the definition Includes both monocotyledons and dicotyledons and all photosynthetic organisms.

[0011] The term 'HPV polypeptides' Is intended to encompass HPV L1 protein; a chimaeric HPV L1 peptide fused to another HPV antigen peptide; a chimaeric HPV L1 peptide fused to a heterologous peptide derived from any antigenic epitope, B-cell or T-cell specific and a HPV L2 protein or their functional equivalents. The same applies, *mutatis mutandis* to the term 'HPV gene'.

[0012] The term 'plant components' Is Intended to encompass plastids, endoplasmic reticuli, cytoplasm and apoplasts, Preferably the plant component is a plastid.

[0013] By targeting It is intended that targeting sequences may be included in the vector. Such targeting sequences may be translated Into a peptide which directs the vector or product thereof to the desired component In the plant, such as a plastid.

[0014] The vector according to the present Invention preferably Includes promoters and other necessary regulators such as terminators or the like operably linked to the coding sequence.

[0015] It will be appreciated that Infiltrating at least a portion of the plant with the vector may give rise to transient expression of the protein and transforming plant tissue with the vector so as to create a transgenic plant may give rise to transgenic expression of the protein, both of which forms of expression are contemplated to fall within the ambit of the present application.

[0016] In this specification reference to a protein, peptide or a gene or their functional equivalents includes references to variants thereof which are capable of performing largely the same function as the protein, peptide, polypeptide or gene. Preferably this Invention relates to the production of the HPV-16 L1 protein; a chimaeric HPV L1 peptide fused to another HPV antigen peptide; or a chimaeric HPV L1 peptide fused to a heterologous peptide derived from any antigenic epltope, B-cell or T-cell specific,

[0017] Preferably the vectors are binary vectors, more preferable *Agrobacterium tumefaciens* binary vectors.

[0018] The vector may be adapted to target plastids by causing an expressed polypeptide to Include a portion capable of Interacting with the thylakold membranes of the plastids, in particular the transfer mechanism of the thylakold membranes. This interaction may cause the polypeptide to be imported into the plastid from the cytoplasm where it is expressed. The mechanism of importation Into the cytoplasm may be important for proper folding of the proteins.

[0019] However, it will be appreciated that the vector may be adapted to target the plastids themselves to become transformed and expression of the polypeptide may occur wholly within the plastid.

[0020] Without wishing to be bound by theory, the applicant is of the view that a polypeptide imported into the plastid Is imported in its primary structure and undergoes folding into its secondary and tertiary structure within the plastid, i.e. remote from the cytoplasm of the cell.

[0021] Preferably the HPV L1 gene; chimaeric HPV L1 gene fused to another HPV antigen gene; or a chimaeric HPV L1 gene fused to a heterologous gene derived from any antigenic epitope, B-cell or T-cell specific is an optimized gene, for example, human-coaon optimized, BCG-codon optimized or plant-codon optimized. The HPV L1 gene or genes of the HPV L1 chimeras may also be modified in another manner, for example nuclear localization signal deficient.

[0022] The method according to the present invention may further Include the step of co-infiltration of the plant with a suppressor protein adapted to inhibit post-transcriptlonal gene silencing in a plant. Preferably the suppressor protein is the NSs protein of the tomato spotted wilt virus or the p19 of tomato bushy stunt virus. Most preferably the suppressor protein is NSs.

[0023] There is described herein plastids selected from chloroplasts, chromoplasts and leucoplasts. The plastids targeted in the present invention are chloroplasts.

[0024] The Infiltration can be done by direct injection or by vacuum. According to one aspect of the present Invention, whole plants may be vacuum infiltrated. In this specification, 'whole plants' should be considered to include its plants which have had their roots removed as well as plants which have been partially defoliated as well as largely intact plants. Reference to plant should Include reference to plant parts including but not limited to seed, leaf, root, stem, flower, fruit, embryo, meristem, hypocotyl, epicotyl, cotyledon, pollen and tissue.

[0025] In the method according to the present invention, both infiltration and transformation of the plant may be achieved with *Agrobacterium tumefaciens* which has been transformed to accept the vector.

[0026] The plant may also be selected from *Nicotiana bentham*/*ana* and *N. tabacum.* It will be appreciated, however, that any plant which supports transient protein expression or being made transgenic will be suitable for the purposes of the present invention.

[0027] Infiltration is preferably performed upon the leaves of the plant. Direct injection infiltration is preferably performed on the abaxial region of the leaf.

[0028] According to the present invention, the method may involve producing HPV L1 polypeptide in a plant wherein substantially the whole plant is infiltrated with a suitable vector by means of vacuum infiltration.

[0029] There is also described herein a HPV L1 polypeptide whenever produced according to a method as hereinbefore described.

[0030] According to another aspect to the present invention there is provided use of a vector into which a HPV L1 gene has been cloned, which vector is adapted to target chloroplasts present in a plant so as to either transiently express an HPV L1 polypeptide in the cytoplasm and then import the HPV polypeptide into the chloroplasts and/or to create a transgenic plant where the HPV L1 polypeptide is expressed in the cytoplasm and then imported into the chloroplasts.

[0031] According to another aspect to the present invention there is provided a vector into which a HPV L1 gene has been cloned, which vector is adapted to target chloroplasts present in a plant so as to either transiently express an HPV L1 polypeptide in the cytoplasm and then import the HPV L1 polypeptide into the chloroplasts and/or to create a transgenic plant where the HPV L1 polypeptide is expressed in the cytoplasm and then imported into the chloroplasts.

[0032] There is also described herein a prophylactic or therapeutic vaccine consisting of HPV L1 1 polypeptide capable of inducing an immunogenic response in a suitable host, whenever produced by a method as hereinbefore described.

[0033] According to another aspect to the present invention there is provided a transgenic plant, part or progeny thereof containing a cell transformed with a vector adapted to target chloroplasts present in the plant and encoding an HPV L1 gene or nucleic acid encoding a functional equivalent thereof, such that an HPV L1 polypeptide is expressed in the cytoplasm and then imported into the chloroplasts.

[0034] In these subsequent aspects to the Invention, the options and preferences of the first aspect apply *mutatis mutandis.*

[0035] A need existed to optimise the expression of commercially viable proteins In plants, to levels that would make plants a viable platform. One example is the production of HPV L1 major capsid protein as capsomeres and/or as virus-like particles (VLPs) for human vaccine or reagent purposes.

[0036] By following the teaching of this invention, the expression of a number of antigenic proteins can be compared, as well as the effect of plant cell compartment targeting of the proteins by transient expression by means of agroinfiltration. This method involves the infiltration of *Agrobacterium tumefaciens* recombinants carrying expression vectors Into plants by direct injection or by creating a vacuum over leaves submerged or soaked In the *Agrobacterium* culture.

[0037] Human-codon optimised or BCG codon optimised HPV L1 was expressed at significantly higher levels than the other L1 sequences and human codon optimisation has also resulted In high expression of influenza H5 protein. Chloroplast-targeting caused significantly higher accumulation than cytoplasmic or endoplasmic reticulum (ER)-targeting. Electron microscopy of whole-plant extracts of HPV L1 revealed that L1 was assembling into virus-like particles (VLPs) in the plants. The protein produced also reacted with appropriate HPV L 1-specifIc monoclonal antibodies (MAbs) which recognise conformation-specific epitopes.

[0038] L1 obtained by transient expression in plants induced high levels of L1-specific antibodies in mice, and these antibodies were found to be neutralising In an *in vitro* neutralisation assay. The expression vectors that facilitated high transient L1 expression in plant chloroplasts were utilised to generate transgenic plants that also expressed high levels of the other polypeptides as hereinbefore described.

[0039] *Agrobacterium* vacuum infiltration typically involves infiltration of leaves that have been removed from the stem. The present invention describes novel vacuum infiltration of intact plants, or intact rootless plants. Intact plants with small root systems can removed from the soil, infiltrated and replanted without loss of viability. Large root systems can be removed, the plant infiltrated, and then replanted in plant foam to be cultured for at least three days without loss of viability. Plants with complex root systems can also be grown hydroponically which permits infiltration without root removal. Whole plants are easier to incubate than loose leaves after the infiltration process, and they survive for longer, thus increasing foreign gene expression. The advantage of infiltrating intact plants is the possibility to scale up foreign protein production by transient expression to levels that are commercially viable.

[0040] The present invention also teaches the targeting of HPV L1 protein to specific plant cell subcompartments (ER and chloroplasts) by agroinfiltration or in transgenic plants. Chloroplast-targeting significantly increased L1 accumulation over ER-targeted and cytoplasmic accumulation. Chloroplast accumulation may protect L1 protein from degradation by proteases present in the cytoplasm and/or allow it to accumulate to higher concentration without affecting the function of the plant cell.

EXAMPLES

Example 1

Expression of HPV L1 protein to high levels

Expression constructs

[0041] Three *Agrobacterium* vectors: pTRAc, pTRAkc-rbcs1-cTP and pTRAkc-ERH (Figure 1) were obtained from Rainer Fischer (Fraunhofer Institute, Aachen, Germany). The pTRAc vector consists of a Cauliflower Mosaic Virus (CaMV) 35S promoter (P35SS), with duplicated transcriptional enhancer, chalcone synthase 5' untranslated region (CHS) and CaMV 35S polyadenylation signal (pA35S), for foreign gene expression; 2 scaffold attachment regions (SAR) for the tobacco RB7 gene; the left and right borders for T-DNA integration; origins of replication for *E. coli* and *Agrobacterium tumefaciens*; and the *bla* gene for antibiotic selection. pTRAkc-rbcs1-cTP is a derivative of pTRAc with an additional rbcs-cTP sequence (chloroplast targeting signal from the small subunit of Rubisco from *Solanum*), forming a 3' fusion with the foreign gene. pTRAkc-ERH is also derivative of pTRAc, and includes the KDEL and his6 sequences, forming a 5' fusion with the foreign gene. The pTRAkc-rbcs1-cTP and pTRAkc-ERH vectors also include the *npt* // gene, for kanamycin resistance in plants.

[0042] Four HPV-16 L1 gene variants were cloned into the above mentioned *Agrobacterium* vectors: (1) a South African isolate, SAL1 (GenBank accession no. AY177679); (2) human-codon optimised L1 (HL1, Figure 2); (3) plant-codon optimised L1 (SYNL1, Figure 3), and (4) nuclear localisation signal deficient, 22 amino acid truncation of HL1, HL1AC22.

[0043] To facilitate directional cloning, restriction enzyme sites were added to the termini of the L1 genes by PCR amplification. SAL1 was amplified with sense primer: 5'-GGACGCGTTAGGT ACATGTCTCTTTGGCTGCCT (SEQ. ID NO.1), and antisense primer: 5'-TCTAGACTCGAGTTACAGCTT ACGTTTTTTGCGTTT (SEQ. ID NO. 2), digested with *Afl* III and *Xho* I and cloned into the same sites of pTRAc, forming pTRA-SAL1; or digested with *Mlu* I and *Xho* I and cloned into the same sites in pTRAkc-rbcs1-cTP, forming pTRACTP-SAL1. SAL1 was also amplified with the above sense primer and antisense primer: 5'-AGCGGCCGC CAGCTTACGTTTTTTGCG (SEQ. ID NO. 3), digested with *Afl* III and *Not* I and cloned into the Nco I and *Not* I sites of pTRAkc-ERH, forming pTRAERH-SAL1.

[0044] SYNL1 was amplified with sense primer: 5'-GGACGCGTGAGATTCATGAGCCTTTGGCTC CCT(SEQ. ID NO. 4), and antisense primer: 5'-ATCTAGACTCGAGTTAGAGCTTCCTCTTCTTCCTCTT(SEQ. ID NO. 5), digested with *Bsp* HI and *Xho* I and cloned into the *Afl* III and *Xho* I sites of pTRAc, forming pTRA-SYNL1; or digested with *Mlu* I and *Xho* I and cloned into the same sites in pTRAkc-rbcs1-cTP, forming pTRACTP-SYNL1. SYNL1 was also amplified with the above sense primer and antisense primer: 5'-AGCGGCCGCGAGCTTCCTCTTCTTCCTCTT(SEQ. ID NO. 6), digested with *Bsp* HI and *Not* I and cloned into the *Nco* I and *Not* I sites of pTRAkc-ERH, forming pTRAERH-SYNL1.

[0045] HL1 was amplified with sense primer: 5'-GGACGCGTGAGGTTCATGAGCCTGTGGCTGCC C(SEQ. ID NO. 7), and antisense primer: 5'-ATCTAGACTCGAGTCACAGCTTGCGCTTCTTCCG(SEQ. ID NO. 8), digested with *Bsp* HI and *Xho* I and cloned into the *Afl* III and *Xho* I sites of pTRAc, forming pTRA-HL1; or digested with *Mlu* I and *Xho* I and cloned into the same sites in pTRAkc-rbcs1-cTP, forming pTRACTP-HL1. HL1 was also amplified with the above sense primer and antisense primer: 5'-AGCGGCCGCCAGCTTGCGCTTCTTCCGC(SEQ. ID NO. 9), digested with *Bsp* HI and *Not* I and cloned into the *Nco* I and *Not* I sites of pTRAkc-ERH, forming pTRAERH-HL1.

[0046] HL1ΔC22 was formed by PCR amplification of HL1, with the above sense primer, and the antisense primer: 5' TCTAGACTCGAGTCAGCCCAGGGTGAACTTAGG(SEQ. ID NO. 10), to facilitate termination before the NLS (Zhou et al. 1991). The PCR product was digested with *Mlu* I and *Xho* I and cloned into the same sites in pTRAkc-rbcs1-cTP, forming pTRACTP-HL1ΔC22.

[0047] The 30B-GFPC3 mutant (GeneBank accession no. U62637) *gfp* gene was amplified from pBSG1057 (Biosource Technologies Inc, Vacaville, USA) with sense primer: 5' GGACGCGTT AGGTCCATGGCTAGCAAAGGAGAAG(SEQ. ID NO. 11), and antisense primer: 5' ATCTAGATTATTTGTA GAGCTCATCCATG(SEQ. ID NO. 12), digested with *Nco* I and *Xba* I and cloned into the same sites of pTRAc, forming pTRA-GFP.

Transformation of *Agrobacterium*

[0048] *Agrobacterium* GV3101::pMP90RK (obtained from Rainer Fischer, Fraunhofer Institute, Aachen) was made electrocompetent as described by (Shen and Forde, 1989). 50-200ng of the above described HPV-16 L1/*Agrobacterium* vectors were mixed with 100μl of electrocompetent cells in a 0.1cm electrogap cuvette (BioRad). The cells were transformed in a GenePulser (BioRad) set at 1.8kV, 25μF and 200Ω.

[0049] Electroporated cells were incubated in 1ml LB for 2 hours prior to plating on LB containing 50μg.ml^{-1} carbenicillin, 50μg.ml^{-1} rifampicin and 30.ml^{-1} kanamycin. Positive clones were verified by isolation of plasmid DNA from recombinant

Agrobacterium colonies, and retransformation of plasmid DNA in *E. coli* DHSα cells and selection on 100μg/ml ampicillin.

Table A. Summary of *Agrobacterium* recombinants used

Clone	Vector	Insert	Cell compartment targeted	*Agrobactertum* strain
pTRA-SAL1	pTRAc	SAL1 (South African HPV-16 L1 isolate)	cytoplasm	GV3101
pTRA-SYNL1	pTRAc	SYNL1 (plant-codon optimised HPV-16 L1)	cytoplasm	GV3101
pTRA-HL1	pTRAc	HL1 (human-codon optimised HPV-16 L1)	cytoplasm	GV3101
pTRA-GFP	pTRAc	*Gfp*	cytoplasm	GV3101
pTRACTP-SAL1	pTRAko-rbcsl-cTP	SAL1	plastids	GV3101
pTRACTP-SYNL1	pTRAkcrrbcs1-cTP	SYNL1	plastids	GV3101
pTRACTP-HL1	pTRAkc-rbcs1-cTP	HL1	plastids	GV3101
pTRACTP-HL1AC22	pTRAko-rbcsi-cTP	HLIAC22 (nuclear localisation signal truncation)	plastids	GV3101
pTRACTP-GFP	pTRAko-rbcs1-cTP	*Gfp*	plastids	GV3101
pTRAERH-SAL1	pTRAkc-ERH	SAL1	ER	GV3101
pTRAERH-SYNL1	pTRAko-ERH	SYNL1	ER	GV3101
pTRAERH-HL1	pTRAkc-ERH	HL1	ER	GV31 01
pTRAERH-GFP	pTRAko-ERH	*Gfp*	ER	GV3101
pBIN-NSs	pBIN	*NSs*	cytoplasm	LBA4404

Preparation of *Agrobacterium* for infiltration

[0050] *Agrobacterium* LBA4404 (pBIN-NSs), containing the NSs silencing suppressors gene of TSWV, was obtained from by Marcel Prins (Laboratory of Virology, Wageningen, The Netherlands). *Agrobacterium* GV3101 cultures containing plasmids based on the pTRAc, pTRAkc-rbcs1-cTP and pTRAkc-ERH vectors were supplemented with 50$\mu g.ml^{-1}$ carbenicillin and 50$\mu g.ml^{-1}$ rifampicin. *Agrobacterium* LBA4404 (pBIN-NSs) cultures were supplemented with 50$\mu g.ml^{-1}$ rifampicin and 30$\mu g.ml^{-1}$ kanamycin. *Agrobacterium* cultures were grown shaking at 27°C to log phase ($OD_{600}\approx0.8$) in LB broth, containing the appropriate antibiotics. The cells were collected by centrifugation at 4000g, resuspended in induction medium (LB broth at pH 5.6 containing 10mM 2-[N-morpholino]ethanesulfonic acid [MES], 20mM acetosyringone, and 2mM $MgSO_4$ with the appropriate antibiotics, and grown as above. The cells were collected by centrifugation, as above, and resuspended in infiltration medium (10mM $MgCl_2$, 10mM MES, 2% sucrose and 150$\mu g.ml^{-1}$ acetosyringone, pH 5.6). For vacuum infiltration $MgCl_2$ was replaced with 4g MS salts.l^{-1}. The *Agrobacterium* suspensions were diluted in infiltration medium to OD_{600} 1.0, and were kept at 22°C for 2-3h.

Agroinfiltration

[0051] Injection: The *Agrobacterium*-L1 and *Agrobacterium* (pBIN-NSs) suspensions were diluted and combined in infiltration medium, both to a final OD_{600} of 0.25. When *Agrobacterium* (pTRA-GFP) was co-infiltrated with the above suspension, it was used at a final OD_{600} of 0.0125. Leaves from 2-4-week-old *N. benthamiana* plants were infiltrated by injecting the *Agrobacterium* suspension into the abaxial air spaces from the underside of the leaf. Six leaves were agroinfiltrated with each agrobacterial mixture (3 plants, 2 leaves/plant). The plants were grown for 3-6 days under conditions of 16h light, 8h dark, 22°C.

[0052] Vacuum: *Agrobacterium* (pTRA-HL1) and *Agrobacterium* (pBIN-NSs) were grown overnight in induction medium. The cells from each culture were combined and resuspended in 1-8 litres of infiltration medium, to a final OD_{600} of 0.25 per culture. Whole *N. benthamiana* plants or whole (with roots removed) *N. tabacum* L. cv. Petite Havana SR1 plants were submerged into the *Agrobacterium* suspension and subjected to a vacuum of -90kPa for 10min, with occasional agitation to release trapped air bubbles. The vacuum was released rapidly (-lOkPa.second-^{1}). *N. benthamiana* plants were replanted in soil. *N. tabacum* plants stalks were placed in water-saturated floral foam. The plants were grown

for 3 days under conditions of 16h light, 8h dark, 22°C.

Protein extraction and detection

[0053] Leaf disks (cap of an Eppendorf tube) were harvested from agroinfiltrated leaves, and ground in 250μl high salt (0.5M NaCl) PBS/leaf disk. The extract was centrifuged at 13 000rpm for 5 min in a desktop centrifuge, supernatant collected, and the centrifugation repeated. Large scale extractions were performed on vacuum-infiltrated *N. tabacum* plants: plants were homogenised with a Waring blender in 2ml extraction buffer (0.25M sodium phosphate, 0.1M sodium metabisulphite, 10mM EDTA, 4% polyvinylpolypyrrolidone [PVPP], pH 7.4)/g plant material. The extract was filtered through 2 layers of cheesecloth, after which the filtrate was centrifuged at 10 000g for 20min. The resultant supernatant was ultracentrifuged at 30 000rpm for 3h, and the resultant pellets were resuspended in PBS, and freeze-dried to reduce volume. Total soluble protein (TSP) concentration was determined with a Bradford assay (Sigma).

[0054] For western blot analysis, plant extracts were incubated at 85°C for 2min in loading buffer (Sambrook *et al.*, 1989), separated on a 10% SDS-PAGE gel, and then transferred onto nitrocellulose membrane by semi-dry electroblotting. L1 protein was detected with H16:J4 monoclonal antibody (1:3000), and then with a goat anti-mouse alkaline phosphatase conjugate (1:10000; Sigma). Detection was performed with NBT/BCIP Tablets (Roche).

[0055] L1 protein was quantified from plant extracts by capture ELISA, modified from the polyvinyl alcohol (PVA)-blocking ELISA method of Studentsov *et al.* (2002). A 96-well microtitre plate was coated with monoclonal antibodies H16.J4 (binds linear HPV-16 L1 epitope) or H16.V5 (binds conformational epitope) for 1h at 37°C, washed and blocked. Plant extract was then added for 1h at 37°C, followed by a washing step and addition of rabbit anti-HPV-16 VLP polyclonal serum (1:1000) for 1h at 37°C. This serum was detected with swine anti-rabbit-HRP conjugate (1:5000; DAKO, Denmark) and 1,2-phenylenediamine dihydrochloride (OPD; DAKO) substrate.

[0056] GFP was detected by Capture ELISA. Plant extracts were diluted in 1 % milk solution (Elite® milk powder in PBS with 0.05% Tween 20 [PBS-T]), and incubated on a Reacti-Bind Anti-GFP coated plate (Pierce) for 1h at 37°C. The plate was washed 4 times with PBS-T, followed by the addition of goat anti-GFP HRP conjugate (1:2000 in 1% milk solution; Abcam) the plate was incubated for 30min at 37°C, and then washed 4 times with PBS-T. TMB substrate (KPL) was utilised for detection.

Electron microscopy

[0057] Plant extract was immunotrapped with H16.V5 antibody (1:50) on carbon coated copper grids. The grids were stained with 2% uranyl acetate and viewed using a JEOL 200CX transmission electron microscope.

Immunisation of mice with plant extract and serum analysis

[0058] Approximately 350g of *N. tabacum* that had been vacuum infiltrated with *Agrobacterium* (pTRA-HL1) and *Agrobacterium* (pBIN-NSs) were extracted as described above and resuspended in 0.spl PBS. BALB/c mice were immunised subcutaneously with 1 00pl of the plant extract (11μg L1), either with the addition of Freund's Incomplete adjuvant (4 mice) or without (5 mice). Control groups were immunised twice with 10μg of baculovirus-produced VLPs, or once with 1μg of baculovirus-produced VLPs. Serum was collected from the eye vein 4 weeks post immunisation.

[0059] A modified PVA-blocking ELISA (Studentsov et a/., 2002) was utilised to detect antibodies to L1 in the mice. A microtitre plate was coated with baculovirus-produced HPV-16 L1 VLPs ($2\mu g.ml^{-1}$), blocked for 2h at 4°C with 0.5% PVA in PBS, and then washed 6 times with PBS. Sera were serially diluted in 0.5% PVA (1:40 to 1:40960) and were incubated on the plate at 37°C for 1hr. After washing 6 times with PBS, rabbit anti-mouse HRP conjugate (1:2000; DAKO) was added for 30min at 37°C. Detection was performed with OPD substrate (DAKO).

[0060] The HPV-16 pseudovirus neutralising antibody assay was performed according to the method of (Pastrana *et al.*, 2004). The plasmids required for the assay were obtained from John Schiller (Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, USA).

Plant transformation and regeneration

[0061] *N. tabacum* L. cv. Petite Havana SR1 leaves were cut into $\pm 1cm^2$ pieces, sterilized in 10% bleach and rinsed in sterile water. Leaf discs were dipped into the relevant recombinant *Agrobacterium* culture and grown on co-cultivation media (Table 2) under constant light for two days. The leaf disks were then placed on fresh regeneration media (Table 2) every two weeks until small shoots appeared (4-6 weeks). Shoots of 1.5-2cm were transferred to rooting media (Table 2). Shoots were incubated until strong root growth was evident, and then transplanted to a soil/vermiculite mixture (2:1), covered with plastic bags and kept away from direct light for three days before being moved to direct light. The transgenic plants were screened by PCR using the Extract-N-Amp Plant PCR kit (Sigma) and L1-specific primers.

Table B: Media for the transformation and regeneration of tobacco. All media contained MS with vitamins (Highveld Biological, South Africa), 1% sucrose and 0.8% agar.

	Co-cultivation	Regeneration	Rooting
α-naphtaleneacetic acid	0.1mg.l^{-1}	0.1mg.l^{-1}	
Benzylaminopurine	1mg.l^{-1}	1mg.l^{-1}	
Cefotaxime		2501mg.l^{-1}	250mg.l^{-1}
Kanamycin		300mg.l^{-1}	100mg.l^{-1}

[0062] The injection method of infiltration proved useful for rapid comparison of numerous *Agrobacterium* constructs, and for the optimisation of infiltration culture concentrations. Western blot analysis of *N. benthamiana* leaf samples (Figure 4), after infiltration with *Agrobacterium* carrying the human-codon optimised HPV-16 L1 gene (HL1), demonstrated the successful expression of the 55kDa L1 monomer. HL1 expression from the pTRA-HL1 1 vector (cytoplasmic localisation of L1) was not detectable after 6 days, unless co-infiltration of *Agrobacterium* (pBIN-NSs) occurred. On the other hand, chloroplast-targeted HL1 (pTRACTP-HL1 vector) produced detectable levels of HL1 without *Agrobacterium* (pBIN-NSs), which were boosted with *Agrobacterium* (pBIN-NSs) co-infiltration.

[0063] The above results were confirmed by capture ELISA (Figure 5). Also, the ELISA established that L1 was assembling into the multimeric structures necessary for the generation of neutralising antibodies, as the H16.V5 mAb utilised for this capture ELISA is specific for a conformational epitope formed by pentamers and VLPs. The formation of VLPs were confirmed by electron microscopy (Figure 6) of a crude extract of a *N. benthamiana* leaf that had been infiltrated with *Agrobacterium* GV31 01 (pTRACTP-HL1).

[0064] Codon optimisation and plant compartment targeting affected L1 accumulation significantly (Figure 7). The levels of SYNL1 and SAL1 accumulation in agroinfiltrated leaves were often low, or undetectable, however, HL1 accumulated to high levels (up to ~600mg of L1/kg leaf material; -17% of TSP). It was surprising that plant-codon optimised L1 (SYNL1) was expressed at lower levels than native L1 (SAL1). Targeting of L1 to the chloroplasts increased its accumulation significantly over both cytoplasmic and ER-targeted L1, and may be a result of protection from proteases present in the cytoplasm. L1 expression was lowest when it was targeted to the ER. *Agrobacterium* (pTRA-GFP) was utilised to measure infiltration efficiency deviations between leaves (which were generally found to be small, with a 14% difference between the most and least efficient infiltrations).

[0065] We demonstrated the potential for medium or possibly large-scale expression of HPV-L1 in *N. benthamiana* and *N. tabacum* L. cv. Petite Havana SR1 by vacuum infiltration. Vacuum infiltration of whole *N. benthamiana* plants readily occurred over 90-100% of leaf area, and over 70-90% of *N. tabacum* leaf area. By vacuum infiltration of whole plants, instead of loose leaves as described by Kapila *et al.* (1997), it was relatively easy to scale up infiltration to 2kg of *N. tabacum* plant material.

[0066] A single immunisation of BALB/c mice with a crude *N. tabacum*/HL1 extract induced high HPV-16 VLP-specific antibody titres (40 960) (Figure 8), which were equivalent to that elicited in mice after 2 immunisations with 10μg of baculovirus-produced VLPs. The addition of Freund's Incomplete adjuvant to the plant extract did appear not increase the humoral response elicited to HL1, suggesting that accompanying plant proteins in the sample without Freund's Incomplete adjuvant may have adjuvant properties. An *in vitro* HPV-16 pseudovirus neutralisation assay was performed using the above mouse sera (Table 3). This assay is based on the ability of neutralising serum to block the entry of SEAP HPV-16 pseudoviruses into cells, thus stopping the transfer the SEAP reporter gene into the cells. Mice that had been immunised with the crude *N. tabacum*/HLlextract, successfully elicited neutralising antibody levels greater to those elicited by 2 immunisations with 10μg of baculovirus-produced VLPs. It was surprising that the neutralising antibody levels elicited to the *N. tabacum*/*HL1* extract were lower when Freund's Incomplete adjuvant was added to it.

[0067] *N. tabacum* L. cv. Petite Havana SR1 plants were successfully transformed with *Agrobacterium* carrying the human-codon optimised HPV-16 L1 gene. As was observed with transient expression, chloroplast-targeted L1 accumulated to high levels in the transgenic plants (up to 60mg of L1/kg plant material, 10.6% of the TSP) (Figure 9). The observation that L1 was detected with the conformation-specific H16.V5 mAb from the transgenic leaf extracts suggests that capsomeres and possibly VLP formation is occurring in these plants.

Table C. SEAP HPV-16 pseudovirus neutralisation assay testing the induction of neutralising antibodies in mice, after immunisation with crude plant/HL1 extract. Three days after *N. tabacum* L. cv. Petite Havana SR1 plants were vacuum infiltrated with *Agrobacterium* (pTRA-HL1) and *Agrobacterium* (pBIN-NSs), concentrated plant extracts were produced and used to immunised mice. Sera were taken 4 weeks post-immunisation. Neutralisation titres were defined as the reciprocal of the highest serum dilution that caused at least a 50% reduction in SEAP activity. Titres below 25 were considered negative.

Antigen	Neutralisation (reciprocal of the highest serum dilution to cause at least a 50% reduction in SEAP activity)
PBS	<25
Plant/Agro pTRA-HL1	6400
PlantIAgro pTRA-HL1 + Freund's Inc. ad.	1600
Baculovirus-produced HPV-16 VLP 1μg	<25
Baculovirus-produced HPV-16 VLP 2 x 10μg	1600

Example 2

H5 HA protein of influenza virus expressed to high levels

Plasmid construction

[0068] The full-length gene HA gene (H5, 1704 bp, Figure 10) of the Influenza AlViet Nam/1194/2004 (H5N1) virus (GenBank accession no. AY651333) and a 23 amino acid-truncated HA gene (H5tr, 1635 bp, Figure 11) were human codon optimised, and synthesised by Geneart (Germany). H5tr was truncated from nucleotide 1597-1665 to remove its membrane anchoring domain (Figure 12): this should prevent the HA protein from being associated with cell membranes, and should allow the H5tr protein to be secreted from plant cells after appropriate processing in the ER, which may greatly assist its purification. Enzyme recognition sequences were added during the gene synthesis to facilitate cloning.

[0069] The H5 and H5tr genes were each cloned into the three *A. tumefaciens* vectors, in Example 1. The H5 and H5tr genes were digested with Nco I and Xba I and cloned into the Afl III and Xba I sites of pTRAc, forming the clones pTRA-H5 and pTRA-H5tr. For cloning into pTRAkc-ERH, both vector and H5/H5tr genes were digested with Nco I and Not I, forming pTRAERH-H5 and pTRAERH-H5tr. The H5 and H5tr genes were digested with Mlu I and Xba I and cloned into pTrakc-rbcs1-cTP, forming the clones pTRACTP-H5 and pTRACTP-H5tr. A fourth set of clones, pTRAa-H5 and pTRAa-H5tr, was constructed by cloning the H5 and H5tr genes (digested with Nco I and Xba I) into the pTRAkc-ERH vector after Nco I and Xba I digestion, thus removing the ER retention signal (SEKDEL) to create apoplastic-targeting constructs.

Agrobacterium transformation

[0070] *Agrobacterium* transformation was performed as in example 1.

Agroinfiltration

[0071] Recombinant *A. tumefaciens* GV3101 cultures containing the H5 and H5tr binary vector clones, and *A. tumefaciens* GV3101 (pTRA-P19), containing the p19 silencing suppressor gene of tomato bushy stunt virus, were grown shaking at 27°C to log phase (OD_{600}=0.8) in LB broth supplemented with 50 $\mu g.ml^{-1}$ carbenicillin, 50 $\mu g.ml^{-1}$ rifampicin and 30 $\mu g.ml^{-1}$ kanamycin. The cells were collected by centrifugation at 4000 g, resuspended in induction medium (LB broth at pH 5.6 containing 10 mM MES, 20 μM acetosyringone, and 2 mM $MgSO_4$) with the appropriate antibiotics, and grown as above. The cells were collected by centrifugation, as above, and resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, 2% sucrose and 150 $\mu g.ml^{-1}$ acetosyringone, pH 5.6). The A. *tumefaciens* suspensions were diluted in infiltration medium to OD_{600} 1.0, and were kept at 22°C for 2-3 h.

[0072] *A. tumefaciens-H5* or *A. tumefaciens*-H5tr suspensions were combined with A. *tumefaciens* (pTRA-P19) and diluted with infiltration medium to a final OD_{600} of 0.25 for each culture. Leaves from *N. benthamiana* plants were infiltrated by injecting the bacterial suspension into the abaxial air spaces from the underside of the leaf. Four leaves were agroinfiltrated with each agrobacterial mixture. The plants were grown for 6 days under conditions of 16h light, 8h dark, 22°C.

Protein extraction and HA detection

[0073] *N. benthamiana* leaf disks (cut using the cap of a 2ml microfuge tube) were harvested from agroinfiltrated leaves, and ground in 250 μl high salt phosphate buffer (0.5 M NaCl)/disk. The extract was centrifuged at 13 000 rpm for 5 min, supernatant collected, and the centrifugation repeated.

[0074] The samples were tested for presence of HA by western blot analysis. Plant extracts were incubated at 85 °C for 2 min in loading buffer {Sambrook, 1989 33369 /id}, separated on a 10% SDS-PAGE gel, and then transferred onto nitrocellulose membrane by semi-dry electroblotting. HA protein was detected with H5 positive chicken serum (1:000; James Kitching, Elsenburg, Western Cape Dept of Agriculture), rabbit anti-chicken serum (1:1000; Ed Rybicki, UCT), and then with swine anti-rabbit alkaline phosphatase conjugate (1:10 000; Sigma). Detection was performed with NBT/BCIP Tablets (Roche).

[0075] Agroinfiltration proved useful to rapidly compare the expression of numerous HA constructs in plants. Preliminary results show that the highest accumulation of HA protein was obtained when using the pTRAERH-H5 and pTRAERH-H5tr constructs, which target protein to the ER (Figure 13). HA could not be detected the when using the other constructs.

Example 3

Plasmid Construction

[0076] *Agrobacterium* vectors pTRAc, pTRAkc-ERH,pTRAkc-A, and pTRAkc-rbcs1-cTP (Fig. 14) were supplied by Rainer Fischer (Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Aachen, Germany). Restriction enzyme sites were included at either end of the L2 ORF by polymerase chain reaction (PCR), to facilitate directional cloning into the three *Agrobacterium* vectors. The wild type HPV-16 L2 1.4 kb open reading frame (ORF) (saL2), the HPV-16 L2 ORF codon optimized for expression in plants (plantised) and the codon optimized HPV-16 L2 ORF for mammalian expression (humanised) were amplified by PCR. Codon optimization for *Nicotiana* of HPV-16 L2 (pL2) was done by GENEART® (Regensberg, Germany). The humanised HPV-16 L2 (hL2) was supplied by Martin Müller (Germany).

[0077] The pTRA vectors hold a number of characteristics that optimize the expression of the foreign gene. pTRAc contains the skeleton features and is proficient at transgene expression in the cytoplasm of plant cells (Fig. 14). The pTRAkc-ERH vector includes all the characteristics of pTRAc with a few additions (Fig. 14). A kanamycin resistance gene (*nptII*) was added, this allows these vectors to be used for the generation of transgenic plants. In this study however, transient expression was utilised. pTRAkc-ERH also includes sequences resulting in the carboxy terminal fusions of a His tag and endoplasmic reticulum (ER) retention signal (KDEL). The KDEL sequence is thought to retard proteins at the ER, where they may be protected from degradation. The KDEL sequence may infer protection against degradation as proteins that are unable to form their final conformation remain in the ER (1). Proteins present in the ER that do not have a KDEL sequence are degraded (1). The pTRAkc-A vector is derived from the pTRAkc-ERH vector, it contains the secretory signal 'LPH' but lacks the 'KDEL', thus the protein is secreted out of the cell into the apoplastic space. The pTRAkc-rbcs1-cTP vector which is based on pTRAc, also includes the short stroma-targeting domain (STD) sequence, rbcs1-cTP, which forms an amino terminal STD fusion with the foreign protein. Proteins probably cross where the inner and outer membranes meet, once within the stroma the STD is processed by a 140 kDa metalloprotease (2).

[0078] The left and right borders flank the region of DNA that will be transferred to the plant cell, the T-DNA. The scaffold attachment regions, also known as matrix attachment regions, are on either side of the transgene. These regions are thought to interact with nuclear matrix proteins forming loop domains in DNA which has been shown to increase expression with insertion in a plasmid (3). Expression of the transgene is controlled by a dual P35S CaMV promoter, with a duplicated transcriptional enhancer. This promoter has been shown to increase expression over the traditional single P35S CaMV (4). The polyadenylation signal of the P35S gene is fused to the end of the foreign gene; this stabilizes RNA transcripts. Replication of the plasmid in *Agrobacterium* is initiated at the RK2 origin of replication. Due to the low copy number of the plasmid in *Agrobacterium,* the vector can alternatively be replicated in *Escherichia coli* using the ColE1 origin of replication (5). The development of resistance by a number of *Agrobacterium* to ampicillin has lead to the fusion of carbenicillin and ampicillin resistance in the *bla* gene. Ampicillin is used as a marker in *E. coli* with carbenicillin as a marker in *Agrobacterium.*

[0079] Cloning of all genes into their respective vectors was done using the applicable primer pair. The saL2 ORF was amplified with the primer pair F L2-ERH and R L2-pTRAc (Table 1). The primer pair F pL2-pTRAc and R pL2-pTRAc was used to amplify the pL2 ORF, and the F-hL2-pTRAc and R hL2-pTRAc pair were used to amplify the hL2 ORF to facilitate cloning into pTRAc. The 1.4 kb fragments were cloned into pGEM®-T easy (PROMEGA) and sequenced to confirm PCR fidelity. saL2 was excised with restriction enzymes BspHI and XbaI and subcloned into the AflIII and XbaI sites of pTRAc making plasmid pTRA-saL2-C. Restriction enzymes BspHI and BamHI were used to excise pL2 which was subcloned into the AflIII and BamHI sites of pTRAc making plasmid pTRA-pL2-C. hL2 was cloned using restriction

enzymes BspHI and XbaI to make plasmid pTRA-hL2-C.

[0080] In order to clone saL2 into pTRAkc-ERH, the saL2 ORF was amplified with the primer pair F L2-ERH and R L2-ERH (Table 1), and cloned into the NcoI and NotI sites of pTRAkc-ERH using BspHI and NotI, making plasmid pTRA-saL2-E. Similarly the hL2 ORF was cloned into pTRAkc-ERH using the primer pair F hL2 pTRAc and R hL2-pTRA E.

TABLE 1. Primers used in PCR with their sequence and the restriction sites they attach

Primer Name	Sequence	Underlined Restriction Site
F L2-ERH	GCTCATGAGACACAAACGTTCTGCAAAAG	BspHI
R 1.2-ERH	AAGCGGCCGCGGCAGCCAAAGAGACATC	NotI
R L2-pTRAc	AATCTAGACTAGGCAGCCAAAGAGAC	XbaI
F L2-Plast	AAACGCGTTAGGTGCATGAGACACAAACGTTCTGCAAAAC	MluI
F pL2-pTRAc	AATCATGAGACATAAGAGATCTGCTAAG	BspHI
R pL2-pTRAc	CGGGATCCCTAAGCAGCAAGAGAAACATC	BamHI
F pL2-cTP	GGACGCGTTAGGTGCATGAGACATAAGAGATCTGC	MluI
F hL2-pTRAc	ATTCATGAGGCACAAGAGGAGCGCC	BspHI
R hL2-pTRAc	ATTCTAGATCAGGCGGCCAGGCTCAC	XbaI
F hL2-pTRA-P	ATACGCGTTAGGTGCATGAGGCACAAGAGGAGC	MluI
R hL2-pTRA-E	ATGCGGCCGCGGCGGCCAGGCTCACGTC	NotI

Sequences highlighted in red indicate the start codon of the L2 ORF.
Sequences highlighted in blue indicate the stop codon of the L2 ORF.

[0081] For cloning into pTRAkc-rbcs1-cTP, saL2, pL2 and hL2 were PCR amplified with the primer pairs F L2-Plast and R L2-pTRAc, F pL2-cTP and R pL2-pTRAc, and F hL2-pTRA-P and R hL2-pTRAc respectively (Table 1). hL2 and saL2 were further cloned using enzymes MluI and XbaI into the MluI and XbaI sites of pTRAkc-rbcs1-cTP, forming the plasmid pTRA-saL2-P and pTRA-hL2-P. MluI and BamHI were used to clone pL2 into the MluI and BamHI sites of pTRAkc-rbcs1-cTP, creating pTRA-pL2-P.

[0082] The pTRA-hL2-A clone was made by cloning the same BspHI and XbaI fragment used to make pTRA-hL-C and cloned into the BspHI - XbaI fragment of pTRAkc-ERH Generation of Recombinant *Agrobacterium*

[0083] *Agrobacterium tumefaciens* GV3101 was provided by Rainer Fischer (Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Aachen, Germany). The GV3101 strain contains the helper plasmid, pMP90RK, which contains the crucial *vir* genes (5). The pTRA vectors, as mentioned previously, can be replicated in *E.coli* as well as in *Agrobacterium.* The vector constructs were first cloned into DH5α cells, which are easier to culture and have a higher copy number of the plasmid in comparison with *Agrobacterium* (5). *Agrobacterium* GV3101 cells were grown to log phase 0.8 OD_{600} at 26 °C with shaking, in Luria broth (LB) containing antibiotics; 50 μg/ml rifampicin (Rif) and 30 μg/ml kanamycin (Kan). Cells were made electrocompetent by washing 3 times with Milli-Q water and resuspended in 1/20 the culture volume with 10 % glycerol. Plasmid DNA concentration isolated from DH5α cells was determined by UV spectrometry at 260 nm. Plasmid DNA (400 ng) was mixed with 100 μl of electro-competent GV31 01 cells in a 0.1 cm cuvette (BIORAD®), and electroporated using the following parameters 200Ω, 25μF, and 1.5 kV (Gene Pulse, BIORAD®). After incubation at 26 °C in Luria broth (LB) for 1 hour the electroporated cells were plated on Luria agar (LA), containing 50 μg/ml Rif, 30 μg/ml Kan, and 50 μg/ml carbenicillin (Carb) and grown at 26 °C for 3-4 days.

[0084] Successful transformation was determined either by colony PCR, or by restriction enzyme analysis. Due the low plasmid copy number in *Agrobacterium,* the plasmid from successfully electroporated GV3101 colonies were extracted and transformed into *E. coli* to obtain sufficient DNA for restriction analysis. This was accomplished by inoculating LB containing Rif, Kan, and Carb with *Agrobacterium* colonies which had grown at 26 °C on the luria agar. Competent DH5α cells were transformed with isolated plasmid DNA from the *Agrobacterium* and plated on LA with 100 μg/ml ampicillin incubated at 37 °C O/N. Five DH5α colonies for each viable *Agrobacterium* colony were used to inoculate LB containing 100 μg/ml ampicillin grown at 37 °C to log phase. Cultures were used for small-scale plasmid DNA preparation and restriction digest analysis was undertaken to determine if the insert was present. Glycerol stocks of the suitable *Agrobacterium* colony were made. nine L2 *Agrobacterium* GV3101 strains were made containing the plasmids: pTRA-saL2-E, pTRA-saL2-C, pTRA-saL2-P, pTRA-pL2-C, pTRA-pL2-P, pTRA-hL2-A, pTRA-hL2-C, pTRA-hL2-E, and pTRA-hL2-P (Table 2).

Plant Lines

[0085] Wild type *Nicotiana benthamiana* were grown under conditions of 16h light, 8h dark at 22°C. Plants were utilised

14-28 days after being transplanted from seedling trays to pots.

Preparation of *Agrobacterium* for Infiltration

[0086] The infiltration protocol simulates the natural process of *Agrobacterium* induction by acetosyringone. The induction and infiltration medium both contain acetosyringone which activates the various *vir* genes (6). *Agrobacterium* strains were grown from glycerol stocks in LB with Rif, Kan, and Carb to an OD_{600} between 1 and 2 at 26 °C. *Agrobacterium* was centrifuged at 5,000xg for 10 min at room temperature (RT) and resuspended in induction medium (LB, 10 mM 2-[N-morpholino]ethanesulfonic acid [MES], 2 mM $MgSO_4$, 20 μM acetosyringone, pH 5.6, with Rif, Carb, and Kan) and grown to an OD_{600} of between 1 and 2 at 26°C. Cells were centrifuged at 5,000xg for 10 min at RT and resuspended in infiltration medium (10 mM MES, 10 mM $MgCl_2$, 2.2105 g/l Murashige Skoog [MS] salts, 35 g/l sucrose, 150 μM acetosyringone, at pH 5.6). The optical density of the cells were measured and diluted to an OD_{600} of between 0.4 and 1.0. The cells were incubated for 3 h in the infiltration medium before infiltration.

TABLE 2. A summary of *Agrobacterium* strains, the vectors that were used, the plasimds made, the inserts they contain and where the heterologous protein is targeted

Agrobacterium Strain	*Agrobacterium* Vector	Plasmid Name	Insert	Cell Compartment Targeted
GV3101	pTRAc	pTRA-saL2-C	HPV-16 saL2	cytoplasm
GV3101	pTRAkc-ERH	pTRA-saL2-E	HPV-16 saL2	endoplasmic reticulum
GV3101	pTRAkc-rbcs1-cTP	pTRA-saL2-P	HPV-16 saL2	chloroplast
GV3101	pTRAc	pTRA-pL2-C	HPV-16 pL2	cytoplasm
GV3101	pTRAkc-rbcs1-cTP	pTRA-pL2-P	HPV-16 pL2	chloroplast
GV3101	pTRAc	pTRA-hL2-C	HPV-16 hL2	cytoplasm
GV3101	pTRAkc-A	pTRA-hL2-A	HPV-16 hL2	apoplastic space
GV3101	pTRAkc-ERH	pTRA-hL2-E	HPV-16 hL2	endoplasmic reticulum
GV3101	pTRAkc-rbcs1-cTP	pTRA-hL2-P	HPV-16 hL2	chloroplast
*GV3101	pTRAc	pTRA-HL1	HPV-16 HL1	cytoplasm
*GV3101	pTRAc	pTRA-p19	p19	cytoplasm

**Agrobacterium* strains supplied by James MacLean
HPV-16 saL2 - wild type L2 ORF.
HPV-16 pL2 - L2 ORF optimized for expression in *Nicotiana.*
HPV-16 saL1b - wild type L1 ORF.
HPV-16 hL1 - L1 ORF optimized for expression in *Homo sapiens.*

Infiltration Procedure

[0087] A mammalian optimized HPV-16 L1 gene in GV3101 cloned into the cytoplasmically targeted vector (pTRA-hL1) was kindly given by Dr. James MacLean (UCT, Cape Town, South Africa) *Agrobacterium* GV3101 strains containing L2 or L1 constructs were either infiltrated alone or mixed with equal amounts of GV3101 (pTRA-p19) which codes for a silencing suppressor p19, this strain was also kindly donated by Dr. James MacLean (UCT, Cape Town, South Africa). Two different infiltration protocols were used; injection infiltration or infiltration under a vacuum. For injection infiltration a 2 ml syringe was used to inject the *Agrobacterium* suspended in infiltration medium into the abaxial air spaces of *N. benthamiana* leaves.

[0088] For vacuum infiltration whole *N. benthamiana* plants were suspended in infiltration medium containing the *Agrobacterium* strains and placed under vacuum at 60 mbar for 5 min. *Agrobacterium* was infiltrated with the release of the vacuum. The protocol differs to that used by Vaquero *et al.* (1999) (7). Whole plants were uprooted, vacuum infiltrated and replanted. Vaquero *et al.* (1999) vacuum infiltrated whole leaves of Petite Havanna plants. Use of whole plants is not only easier but additional nutrients may be given to the leaves to increase transgene expression. Plants are subsequently incubated at 28°C in a humidity-controlled room.

Protein Extraction and Western Blots

[0089] Homogenization was done by grinding the material in liquid nitrogen. Homogenized sample was suspended in 2 μl/mg of 8M urea. Cell debris and other larger molecules were separated by two centrifugation rounds (10,000xg, 10 min at RT). Sodium dodecyl sulphate (SDS)-PAGE loading buffer was added to the samples and boiled for 10 min. Sample was loaded onto a 10% SDS-PAGE and run at 100 V for ~2.5 h. Protein was transferred to nylon membrane (Nitrobind, Cast, Pure nitrocellulose, 0.45 micron, OSMONICS INC.) by semi-dry blotting at 15 V, 400 mA, for 2h (Trans Blot® semi-dry, BIORAD® with the power supply Electrophoresis Power Supply, AMERSHAM®). The success of transfer was measured by coomassie blue staining of the gel after transfer. The membrane was blocked O/N in 5% skimmed milk suspended in PBS with 0.05% Tween-20. The membrane was incubated for a further 4 hours at RT with rabbit polyclonal antiserum raised against HPV-16 L2 (1:3,000), mouse monoclonal antibodies against HPV-16 L1 (J4) (1:5,000). Membranes were incubated with goat-anti-rabbit or goat-anti-mouse antibodies conjugated with alkaline phosphatase (SIGMA®-Aldrich) diluted to 1:10,000 for 2h at RT. Immunodetection was done using 5-Bromo-4-Chloro-3-indolyl phosphate / Nitroblue tetrazolium (BCIP/NBT) made according to manufacturer instructions (ROCHE® Diagnostics).

Expression of L2 by transient expression in *N. benthamiana* plants.

[0090] *N. benthamiana* plants were infiltrated with all nine *Agrobacterium* strains containing the different L2 ORFs. Expression of L2 was unable to be detected by western blot any of the strains containing the pL2 ORF or the saL2 ORF (data not shown). This could be due to codon usage as has been previously published for L1. (8) Expression was however noted when expressed from any of the clones having the hL2 gene (Fig. 15). Similar expression of L2 across the suite of vectors was noted. Similar amounts of degradation products were also noted in the different vectors. This possibly indicates that L2 is localized to the same region of the plant cell despite signalling molecules within the vector. A certain degree of protection particularly by the chloroplast-targeted proteins would be expected. These figures contradict other findings made with these vectors, for example, L1 expression is markedly less with the ER-targeted vector than that of the chloroplast- or cytoplasmic-targeted vectors. (Dr. James MacLean, personal communication)

L2 appears to be highly expressed.

[0091] Plant material from each infiltration was weighed and thus the same amount of buffer (8M urea) was added. This allows the possibility of empirically determining the original weight of plant material that was loaded on a western (Fig. 3). This immunoblot shows that L2 can still be detected up to 0.4 mg of original plant material. We can only speculate the amount of L2 that is present relative to total soluble protein, further analysis by ELISA was attempted but unsuccessful due to only a polyclonal antibody being available.

Co-expression of L1 and L2 in the same region.

[0092] L2 and L1 were simultaneously co-infiltrated into *N. benthamiana* plants. Expression of both L1 and L2 was able to be detected in these leaf samples, however from these results it is inconclusive whether L1 and L2 are being expressed in the same cell. The literature suggests that they are more than likely being expressed in the same cell, with expression of antibodies with multiple subunits that form, having been proven. (29)

[0093] Immune responses against L1 have been shown to produce type-specific antibodies, with the advent of the GSK and Merck HPV vaccines this has paved the way for second generation vaccines that will more than likely incorporate L2 and its cross-neutralizing properties. The production of heterologous proteins and in particular subunit vaccines in plants has been shown to be more cost effective than other expression systems. In conclusion a marriage of these two simple yet important ideals could lead to what is needed most in the fight against cervical cancer: a cheap and effective vaccine.

Example 4

Chimaeric HPV L1 protein expressed to high levels

[0094] The method described in W02003/097673, which document is incorporated herein by reference, is used for high level expression of chimaeric HPV L1 proteins as hereinbefore described.

FIGURES

[0095] The specification should be read with reference to the following figures in which:

Figure 1 shows *Agrobacterium* vectors pTRAc, pTRAkc-rbcs1-cTP and pTRAkc-ERH.

Figure 2 shows Human-codon optimised HPV-16 L1 (HL1) (SEQ. ID NO. 13).

Figure 3 shows Plant-codon optimised HPV-16 L1 (SYNL1) (SEQ. ID NO. 14).

Figure 4 shows a western blot of *N. benthamiana* leaf samples after infiltration with *Agrobacterium* carrying the HPV-16 L1 gene. *N. benthamiana* leaves were infiltrated by injection with an *Agrobacterium-L1* construct, or co-infiltrated with *Agrobacterium-L1* and *Agrobacterium* (pBIN-NSs). The western blot was performed on crude leaf extracts 6 days post infiltration, using H16.J4 anti-HPV-16 L1 monoclonal antibody. The samples in lanes 1-5 were from leaves infiltrated with *Agrobacterium* carrying the following vectors: 1, pTRACTP-GFP; 2, pTRA-HL1; 3, pBIN-NSs and pTRA-HL1; 4, pTRACTP-HL1; 5, pBIN-NSs and pTRACTP-HL1.

Figure 5 shows detection of L1 in *N. benthamiana* leaf samples after infiltration with *Agrobacterium* carrying the HPV-16 L1 gene. *N. benthamiana* leaves were infiltrated by injection with an *Agrobacterium-L1* construct, or co-infiltrated with *Agrobacterium-L1* and *Agrobacterium* (pBIN-NSs). Six days post infiltration crude leaf extracts were assessed by a H16.V5 capture ELISA.

Figure 6 shows electron micrograph of crude *N. benthamiana* plant extract after infiltration with *Agrobacterium* GV3101 (pTRACTP-HL1). VLPs are indicated by the arrows.

Figure 7 shows milligrams of HPV-16 L1 produced in plants by agroinfiltration. N. *benthamiana* leaves were infiltrated a mixture of an *Agrobacterium-L1* construct, *Agrobacterium* (pBIN-NSs), and *Agrobacterium* (pTRA-GFP). Six days post infiltration crude leaf extracts were assessed by H16.V5 and H16.J4 mAb capture ELISAs. The L1 level expressed as a % of the total soluble protein (TSP) is displayed for specific constructs.

Figure 8 shows HPV-16 VLP-specific systemic antibody titres induced in BALB/c mice after a single immunisation with a crude plant/HL1 extract. Three days after *N. tabacum* L. cv. Petite Havana SR1 plants were vacuum infiltrated with *Agrobacterium* (pTRA-HL1) and *Agrobacterium* (pBIN-NSs), concentrated plant extracts were produced, and used to immunised mice. Control groups were immunised twice with 10μg of baculovirus-produced VLPs, or once with 1μg of baculovirus-produced VLPs. Sera were taken 4 weeks post-immunisation, serially diluted (pooled for each group of mice) and used in an ELISA against HPV-16 VLPs. The OD 492nm values were measured and the results recorded as the reciprocal of the highest dilution where the OD is >2x that of the prebleed.

Figure 9 shows milligrams of HPV-16 L1 produced per kg of transgenic plant material. *N. tabacum* L. cv. Petite Havana SR1 plants were transformed with *Agrobacterium* carrying the HPV-16 L1 gene. L1 was detected in crude leaf extracts by H16.V5 and H16.J4 mAb capture ELISAs. The L1 level expressed as a % of TSP is displayed for specific constructs.

Figure 10 shows a human codon optimised full-length gene HA gene (H5, 1704 bp) of the Influenza A/Viet Nam/1194/2004 (H5N1) virus (GenBank accession no. AY651333) (SEQ. ID NO. 15).

Figure 11 shows a 23 amino acid-truncated human codon optimised HA gene (H5tr, 1635 bp). H5tr was truncated from nucleotide 1597-1665 to remove its membrane anchoring domain (SEQ. ID NO. 16).

Figure 12 shows the transmembrane prediction for the H5 gene (CBS Prediction Servers, htta:/lwww.cbs.dtu.dk).

Figure 13 shows the Western blot of *N. benthamiana* leaf samples after infiltration with *Agrobacterium* carrying the H5 or H5tr genes. The *Agrobacterium* samples were as follows: 1, pTRA-H5; 2, pTRA-H5tr; 3, pTRACTP-H5; 4, pTRACTP-H5tr; 5, pTRAERH-H5; 6, pTRAERH-H5tr. Lane 7 contained non-infiltrated *N. benthamiana* extract.

Figure 14 shows *Agrobacterium* vectors utilised in this study, namely pTRAc, pTRAkc-ERH, pTRAkc-rbcs1-cTP, and pTRAkc-A. The light blue bar indicates the T-DNA that is transferred to the plant cell at transfection. The heterologous gene is cloned into the vectors at the multiple cloning site (MCS). The vectors share a number of

common features indicated by regions in grey. The T-DNA region is flanked by the left border (LB) and the right border (RB). At either end of the MCS are scaffold attachment regions (SAR). Expression of the transgene is controlled by a dual 35S Cauliflower Mosaic Virus promoter (P35SS CaMV) with attachment of the polyadenylation signal of the same CaMV gene (pA35S). Replication of the vector in *Agrobacterium* is initiated at the RK2 ori. A separate replication initiation site, CoIE1, is used in *E. coli*. The pTRAc vector contains only one antibiotic resistance marker (*bla*) allowing selection with ampicillin/carbenicillin. The pTRAkc-ERH, pTRAkc-A, and pTRAkc-rbcs1-cTP contain a second antibiotic marker (*nptII*) permitting selection with kanamycin in plants. The *nptII* gene is controlled by the promoter of the *Agrobacterium* gene nopaline synthase (Pnos), the polyadentlation of the same gene is attached to nptII (pAnos). pTRAkc-ERH additionally includes a secretory signal (LPH), His tag (His6) sequence and an endoplasmic reticulum retention signal (KDEL) sequence downstream of the MCS. pTRAkc-rbcs1-cTP includes a chloroplast signalling peptide (rbcs1-cTP) sequence upstream of the MCS.

Fig. 15 shows that expression of L2 can be detected with each of the four vectors. *N. benthamiana* was transiently infiltrated with the same OD of Agrobacterium containing the different vectors that have the humanised L2 gene. These are pTRA-hL2-A (hL2-A), pTRA-hL2-C (hL2-C), pTRA-hL2-E (hL2-E), and pTRA-hL2-P (hL2-P). As a negative control plant material that had not been infiltrated with Agrobacterium were also extracted (-ve). *E. coli*-expressed HPV-16 L2 was used as a positive control (+ve) Plant material was extracted after two days (A) and five days (B) and immunoblotted. Westerns were probed using the rabbit pAb-aL2.

Fig. 16 shows a crude quantification of the amount of L2 present in leaf material after infiltration. *N. benthamiana* was infiltrated with the Agrobacterium strain containing the pTRA-hL2-C vector. Plant material was extracted four days after infiltration. Various amounts of leaf material (8 mg, 0.8 mg and 0.4 mg) were empirically determined and loaded onto a western. Westerns were probed using the rabbit pAb-αL2.

Figure 17 shows L1 and L2 are co-expressed in the same region. *N. benthamiana* was infiltrated with the same OD of Agrobacterium containing the cytoplasmically-targeted humanised L1 gene mixed and co-infiltrated with Agrobacterium containing the humanised L2 gene in the vector also targeting the protein to the cytoplasm. Plant material that was co-infiltrated was extracted after four days and immunoblotted (L1/L2). Uninfiltrated plant material was used as a negative (-ve). *E. coli*-expressed L2 was loaded and run as a positive control. Immunoblots were either probed with mouse mAb J4 (αL1) or with rabbit polyclonal Ab-αL2 (αL2).

[0096] In this specification, where any sequence according to the invention is described or otherwise set out, the invention is intended to encompass sequences of at least 75% homology, more preferably at least 77% homology, even more preferably at least 80% homology, even more preferably at least 82% homology, even more preferably at least 85% homology, even more preferably at least 87% homology, even more preferably at least 90% homology, even more preferably at least 92% homology, even more preferably at least 95% homology, most preferably at least 97% homology.

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[0097]

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Claims

1. A method of producing HPV L1 polypeptides in a plant comprising the steps of:

   - cloning a HPV L1 gene or nucleic acid encoding a functional equivalent into a vector adapted to target a polypeptide expressed in the cytoplasm to the chloroplasts present in the plant;
   - infiltrating at least a portion of the plant with the vector or transforming plant tissue with the vector so as to transiently express the HPV L1 polypeptides in the cytoplasm and then import the HPV L1 polypeptides into the chloroplasts and/or to create a transgenic plant wherein the HPV L1 polypeptides are expressed in the cytoplasm and then imported into the chloroplasts;
   and
   - recovering the HPV L1 polypeptides expressed by the plant.

2. A method according to claim 1 wherein the HPV L1 polypeptides are selected from the group consisting of a HPV L1 protein; a chimaeric HPV L1 peptide fused to another HPV antigen peptide; a chimaeric HPV L1 peptide fused to a heterologous peptide derived from any antigenic epitope, B-cell or T-cell specific, or their functional equivalents.

3. A method according to any previous claim wherein targeting sequences encoding a polypeptide for directing the HPV L1 polypeptide from the cytoplasm to the chloroplast are included in the vector.

4. A method according to any previous claim wherein the vector includes promoters and other regulators or the like operably linked to the coding sequence of the vector.

5. A method according to any previous claim wherein the vectors are binary vectors.

6. A method according to claim 5 wherein the vectors are *Agrobacterium tumefaciens* binary vectors.

7. A method according to claim 2 wherein the HPV L1 gene; chimaeric HPV L1 gene fused to another HPV antigen gene; or chimaeric HPV L1 gene fused to a heterologous gene derived from any antigenic epitope, B-cell or T-cell specific; is a codon-use optimized gene.

8. A method according to claim 7 wherein the optimized gene is human-codon optimized, BCG-codon optimized or plant-codon optimized.

9. A method according to claim 2 wherein the HPV L1 gene or genes of the HPV L1 chimaeras are modified to be

nuclear localization signal deficient.

10. A method according to any previous claim further including the step of co-infiltration of the plant with a suppressor protein adapted to inhibit post-transcriptional gene silencing in a plant.

11. A method according to claim 10 wherein the suppressor protein is the NSs protein of the tomato spotted wilt virus or the p19 of tomato bushy stunt virus.

12. A method according to any previous claim further including the step of co-infiltration of the plant with a HPV L2 gene.

13. A method according to any previous claim wherein the infiltration is done by direct injection or by vacuum.

14. A method according to any previous claim wherein infiltration and/or transformation of the plant is achieved with *Agrobacterium tumefaciens* which has been transformed to accept the vector.

15. A method according to any previous claim wherein the plant is selected from *Nicotiana benthamiana* and *N. tabacum.*

16. A method according to any previous claim wherein infiltration is performed upon the leaves of the plant.

17. A method according to any previous claim wherein direct injection infiltration is performed on the abaxial region of the leaf.

18. A method according to any previous claim wherein the HPV L1 gene or nucleic acid encoding a functional equivalent is selected from the sequences shown in Figures 2 and 3.

19. A method of producing HPV L1 polypeptide in a plant wherein substantially the whole plant is infiltrated with a suitable vector by means of vacuum infiltration.

20. A method according to any one of claims 1 to 19 wherein the vector is adapted to target chloroplasts present in the plant by causing an expressed polypeptide to include a portion capable of interacting with the thylakoid membranes of the chloroplasts.

21. Use of a vector into which a HPV L1 gene has been cloned, which vector is adapted to target chloroplasts present in a plant so as to either transiently express an HPV L1 polypeptide in the cytoplasm and then import the HPV polypeptide into the chloroplasts and/or to create a transgenic plant where the HPV L1 polypeptide is expressed in the cytoplasm and then imported into the chloroplasts.

22. Use according to claim 21 wherein the vector is adapted to target chloroplasts present in the plant by causing an expressed polypeptide to include a portion capable of interacting with the thylakoid membranes of the chloroplasts.

23. A vector into which a HPV L1 gene has been cloned, which vector is adapted to target chloroplasts present in a plant so as to either transiently express an HPV L1 polypeptide in the cytoplasm and then import the HPV L1 polypeptide into the chloroplasts and/or to create a transgenic plant where the HPV L1 polypeptide is expressed in the cytoplasm and then imported into the chloroplasts.

24. A vector according to claim 23 wherein the vector is adapted to target chloroplasts present in the plant by causing an expressed polypeptide to include a portion capable of interacting with the thylakoid membranes of the chloroplasts.

25. A transgenic plant, part or progeny thereof containing a cell transformed with a vector adapted to target chloroplasts present in the plant and encoding an HPV L1 gene or nucleic acid encoding a functional equivalent thereof, such that an HPV L1 polypeptide is expressed in the cytoplasm and then imported into the chloroplasts.

26. A transgenic plant, part or progeny thereof according to claim 25 wherein the vector is adapted to target chloroplasts present in the plant by causing an expressed polypeptide to include a portion capable of interacting with the thylakoid membranes of the chloroplasts.

Patentansprüche

1. Verfahren zur Erzeugung von HPV-L1-Polypeptiden in einer Pflanze, welches die folgenden Schritte umfasst:

   - Klonieren eines HPV-L1-Gens oder einer Nukleinsäure, welche ein funktionelles Äquivalent codiert, in einen Vektor, der dafür ausgelegt ist, ein Polypeptid, das in dem Cytoplasma exprimiert wird, zu den in der Pflanze vorhandenen Chloroplasten zu leiten,
   - Infiltrieren wenigstens eines Teils der Pflanze mit dem Vektor oder Transformieren des Pflanzengewebes mit dem Vektor, um die HPV-L1-Polypeptide in dem Cytoplasma transient zu exprimieren und dann die HPV-L1-Polypeptide in die Chloroplasten zu importieren und/oder um eine transgene Pflanze zu erzeugen, wobei die HPV-L1-Polypeptide in dem Cytoplasma exprimiert und dann in die Chloroplasten importiert werden, und
   - Gewinnen der HPV-L1-Polypeptide, die von der Pflanze exprimiert werden.

2. Verfahren gemäß Anspruch 1, wobei die HPV-L1-Polypeptide ausgewählt sind aus der Gruppe bestehend aus einem HPV-L1-Protein, einem mit einem anderen HPV-Antigen-Peptid fusionierten chimären HPV-L1-Peptid, einem mit einem heterologen Peptid, das aus einem B-Zellen- oder T-Zellen-spezifischen Antigen-Epitop abgeleitet ist, fusionierten chimären HPV-L1-Peptid oder deren funktionellen Äquivalenten.

3. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Zielsteuerungssequenzen, die ein Polypeptid codieren, um die HPV-L1-Polypeptide von dem Cytoplasma zu dem Chloroplasten zu lenken, in dem Vektor enthalten sind.

4. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei der Vektor Promotoren und andere Regulatoren oder dergleichen einschließt, die mit der codierenden Sequenz des Vektors funktionsfähig verbunden sind.

5. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Vektoren binäre Vektoren sind.

6. Verfahren gemäß Anspruch 5, wobei die Vektoren binäre Vektoren von *Agrobacterium tumefaciens* sind.

7. Verfahren gemäß Anspruch 2, wobei das HPV-L1-Gen, das mit einem anderen HPV-Antigen-Gen fusionierte chimäre HPV-L1-Gen, oder das mit einem heterologen Gen aus einem B-Zellen- oder T-Zellen-spezifischen Antigen-Epitop fusionierte chimäre HPV-L1-Gen ein für die Anwendung Codon-optimiertes Gen ist.

8. Verfahren gemäß Anspruch 7, wobei das optimierte Gen Menschen-Codon-optimiert, BCG-Codon-optimiert oder Pflanzen-Codon-optimiert ist.

9. Verfahren gemäß Anspruch 2, wobei das HPV-L1-Gen oder Gene der HPV-L1-Chimären verändert werden, um Kernlokalisierungssignal-defizient zu sein.

10. Verfahren gemäß einem der vorhergehenden Ansprüche, welches ferner den Schritt der Co-Infiltration der Pflanze mit einem Suppressor-Protein umfasst, das dafür ausgelegt ist, die posttranskriptionelle Genstilllegung in einer Pflanze zu hemmen.

11. Verfahren gemäß Anspruch 10, wobei das Suppressor-Protein das NSs-Protein des Tomatenbronzeflecken-Virus oder p19 des Tomatenzwergbusch-Virus ist.

12. Verfahren gemäß einem der vorhergehenden Ansprüche, welches ferner den Schritt der Co-Infiltration der Pflanze mit einem HPV-L2-Gen umfasst.

13. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Infiltration durch Direkteinspritzung oder Vakuum erfolgt.

14. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Infiltration und/oder Transformation der Pflanze mithilfe von *Agrobacterium tumefaciens* erreicht wird, welches transformiert wurde, so dass es den Vektor akzeptiert.

15. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Pflanze ausgewählt ist aus *Nicotiana benthamiana* und *N. tabacum.*

16. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Infiltration an den Blättern der Pflanze ausgeführt wird.

17. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Direkteinspritzungsinfiltration an dem äußeren Bereich des Blattes ausgeführt wird.

18. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei das HPV-L1-Gen oder die Nukleinsäure, die ein funktionelles Äquivalent codiert, ausgewählt ist aus den Sequenzen, die in den Figuren 2 und 3 gezeigt sind.

19. Verfahren zur Erzeugung von HPV-L1-Polypeptiden in einer Pflanze, wobei im Wesentlichen die ganze Pflanze mit einem geeigneten Vektor mittels Vakuuminfiltration infiltriert wird.

20. Verfahren gemäß einem der Ansprüche 1 bis 19, wobei der Vektor dafür ausgelegt ist, auf Chloroplasten zu zielen, die in der Pflanze vorhanden sind, indem er ein exprimiertes Polypeptid dazu veranlasst, einen Bereich zu beinhalten, der mit den Thylakoidmembranen der Chloroplasten interagieren kann.

21. Verwendung eines Vektors, in den ein HPV-L1-Gen geklont wurde, welcher dafür ausgelegt ist, auf Chloroplasten zu zielen, die in der Pflanze vorhanden sind, um entweder ein HPV-L1-Polypeptid in dem Cytoplasma transient zu exprimieren und dann das HPV-Polypeptid in die Chloroplasten zu importieren und/oder eine transgene Pflanze zu erzeugen, bei der das HPV-L1-Polypeptid in dem Cytoplasma exprimiert und dann in die Chloroplasten importiert wird.

22. Verwendung gemäß Anspruch 21, wobei der Vektor dafür ausgelegt ist, auf Chloroplasten zu zielen, die in der Pflanze vorhanden sind, indem er das exprimierte Polypeptid dazu veranlasst, einen Bereich zu beinhalten, der mit den Thylakoidmembranen der Chloroplasten interagieren kann.

23. Vektor, in den ein HPV-L1-Gen geklont wurde, welcher Vektor dafür ausgelegt ist, auf Chloroplasten zu zielen, die in einer Pflanze vorhanden sind, um entweder ein HPV-L1-Polypeptid in dem Cytoplasma transient zu exprimieren und dann das HPV-L1-Polypeptid in die Chloroplasten zu importieren und/oder eine transgene Pflanze zu erzeugen, bei der das HPV-L1-Polypeptid in dem Cytoplasma exprimiert und dann in die Chloroplasten importiert wird.

24. Vektor gemäß Anspruch 23, wobei der Vektor dafür ausgelegt ist, auf Chloroplasten zu zielen, die in der Pflanze vorhanden sind, indem er ein exprimiertes Polypeptid dazu veranlasst, einen Bereich zu beinahlten, der mit den Thylakoidmembranen der Chloroplasten interagieren kann.

25. Transgene Pflanze, ein Teil oder ein Nachkomme davon, enthaltend eine Zelle, die mit einem Vektor transformiert ist, welcher dafür ausgelegt ist, auf Chloroplasten zu zielen, die in der Pflanze vorhanden sind, und der ein HPV-L1-Gen oder eine Nukleinsäure codiert, die ein funktionelles Äquivalent davon codiert, so dass ein HPV-L1-Polypeptid in dem Cytoplasma exprimiert und dann in die Chloroplasten importiert wird.

26. Transgene Pflanze, ein Teil oder ein Nachkomme davon gemäß Anspruch 25, wobei der Vektor dafür ausgelegt ist, auf Chloroplasten zu zielen, die in der Pflanze vorhanden sind, indem er das exprimierte Polypeptid dazu veranlasst, einen Bereich zu beinhalten, der mit den Thylakoidmembranen der Chloroplasten interagieren kann.

Revendications

1. Procédé pour la production de polypeptides HPV L1 dans une plante, comprenant les étapes consistant à :

   - cloner un gène HPV L1 ou un acide nucléique codant pour un équivalent fonctionnel dans un vecteur adapté pour diriger un polypeptide exprimé dans le cytoplasme vers les chloroplastes présents dans la plante ;
   - infiltrer au moins une partie de la plante avec le vecteur ou transformer le tissu de la plante avec le vecteur afin d'exprimer de façon transitoire les polypeptides HPV L1 dans le cytoplasme et importer ensuite les polypeptides HPV L1 dans les chloroplastes et/ou de créer une plante transgénique dans laquelle les polypeptides HPV L1 sont exprimés dans le cytoplasme et importés ensuite dans les chloroplastes ; et
   - récupérer les polypeptides HPV L1 exprimés par la plante.

2. Procédé selon la revendication 1, dans lequel les polypeptides HPV L1 sont choisis dans le groupe constitué par

une protéine HPV L1 ; un peptide HPV L1 chimère fusionné à un autre peptide antigène de HPV ; un peptide HPV L1 chimère fusionné à un peptide hétérologue dérivé d'un épitope antigénique quelconque, spécifique des lymphocytes B ou T, ou leurs équivalents fonctionnels.

3. Procédé selon l'une quelconque des revendications précédentes, dans lequel des séquences de ciblage codant pour un polypeptide pour diriger le polypeptide HPV L1 du cytoplasme vers le chloroplaste sont incluses dans le vecteur.

4. Procédé selon l'une quelconque des revendications précédentes, dans lequel le vecteur comprend des promoteurs et d'autres régulateurs ou similaires liés fonctionnellement à la séquence de codage du vecteur.

5. Procédé selon l'une quelconque des revendications précédentes, dans lequel les vecteurs sont des vecteurs binaires.

6. Procédé selon la revendication 5, dans lequel les vecteurs sont des vecteurs binaires d'*Agrobacterium tumefaciens.*

7. Procédé selon la revendication 2, dans lequel le gène HPV L1 ; le gène HPV L1 chimère fusionné à un autre gène antigène de HPV ; ou le gène HPV L1 chimère fusionné à un gène hétérologue dérivé d'un épitope antigénique quelconque, spécifique des lymphocytes B ou T ; est un gène optimisé quant à l'utilisation des codons.

8. Procédé selon la revendication 7, dans lequel le gène optimisé est optimisé pour les codons humains, optimisé pour les codons du BCG ou optimisé pour les codons des plantes.

9. Procédé selon la revendication 2, dans lequel le gène HPV L1 ou les gènes des chimères HPV L1 sont modifiés pour être dépourvus de signaux de localisation nucléaire.

10. Procédé selon l'une quelconque des revendications précédentes, comprenant en outre l'étape consistant à co-infiltrer la plante avec une protéine suppresseur adaptée pour inhiber la mise sous silence post-transcriptionnelle des gènes dans une plante.

11. Procédé selon la revendication 10, dans lequel la protéine suppresseur est la protéine NSs du virus des tâches bronzées de la tomate ou la protéine p19 du virus du rabougrissement buissonneux de la tomate.

12. Procédé selon l'une quelconque des revendications précédentes, comprenant en outre l'étape consistant à co-infiltrer la plante avec un gène HPV L2.

13. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'infiltration se fait par injection directe ou sous vide.

14. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'infiltration et/ou la transformation de la plante est réalisée avec un *Agrobacterium tumefaciens* qui a été transformé pour accepter le vecteur.

15. Procédé selon l'une quelconque des revendications précédentes, dans lequel la plante est choisie entre *Nicotiana benthamiana* et *N. tabacum.*

16. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'infiltration est réalisée sur les feuilles de la plante.

17. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'infiltration par injection directe est réalisée sur la région abaxiale de la feuille.

18. Procédé selon l'une quelconque des revendications précédentes, dans lequel le gène HPV L1 ou l'acide nucléique codant pour un équivalent fonctionnel est choisi parmi les séquences présentées sur les figures 2 et 3.

19. Procédé pour la production d'un polypeptide HPV L1 dans une plante, dans lequel sensiblement toute la plante est infiltrée avec un vecteur approprié par infiltration sous vide.

20. Procédé selon l'une quelconque des revendications 1 à 19, dans lequel on adapte le vecteur au ciblage des chloroplastes présents dans la plante en amenant un polypeptide exprimé à inclure une partie capable d'interagir avec

les membranes thylakoïdes des chloroplastes.

21. Utilisation d'un vecteur dans lequel un gène HPV L1 a été cloné, ledit vecteur étant adapté pour cibler les chloroplastes présents dans une plante afin d'exprimer de façon transitoire un polypeptide HPV L1 dans le cytoplasme et importer ensuite le polypeptide HPV dans les chloroplastes et/ou de créer une plante transgénique où le polypeptide HPV L1 est exprimé dans le cytoplasme et importé ensuite dans les chloroplastes.

22. Utilisation selon la revendication 21, dans laquelle on adapte le vecteur au ciblage des chloroplastes présents dans la plante en amenant un polypeptide exprimé à inclure une partie capable d'interagir avec les membranes thylakoïdes des chloroplastes.

23. Vecteur dans lequel un gène HPV L1 a été cloné, ledit vecteur étant adapté pour cibler les chloroplastes présents dans une plante afin d'exprimer de façon transitoire un polypeptide HPV L1 dans le cytoplasme et importer ensuite le polypeptide HPV L1 dans les chloroplastes et/ou de créer une plante transgénique où le polypeptide HPV L1 est exprimé dans le cytoplasme et importé ensuite dans les chloroplastes.

24. Vecteur selon la revendication 23, dans lequel on adapte le vecteur au ciblage des chloroplastes présents dans la plante en amenant un polypeptide exprimé à inclure une partie capable d'interagir avec les membranes thylakoïdes des chloroplastes.

25. Plante transgénique, sa partie ou sa descendance, contenant une cellule transformée avec un vecteur adapté au ciblage des chloroplastes présents dans la plante et codant pour un gène HPV L1 ou un acide nucléique codant pour son équivalent fonctionnel, de sorte qu'un polypeptide HPV L1 est exprimé dans le cytoplasme et importé ensuite dans les chloroplastes.

26. Plante transgénique, sa partie ou sa descendance selon la revendication 25, dans laquelle on adapte le vecteur au ciblage des chloroplastes présents dans la plante en amenant un polypeptide exprimé à inclure une partie capable d'interagir avec les membranes thylakoïdes des chloroplastes.

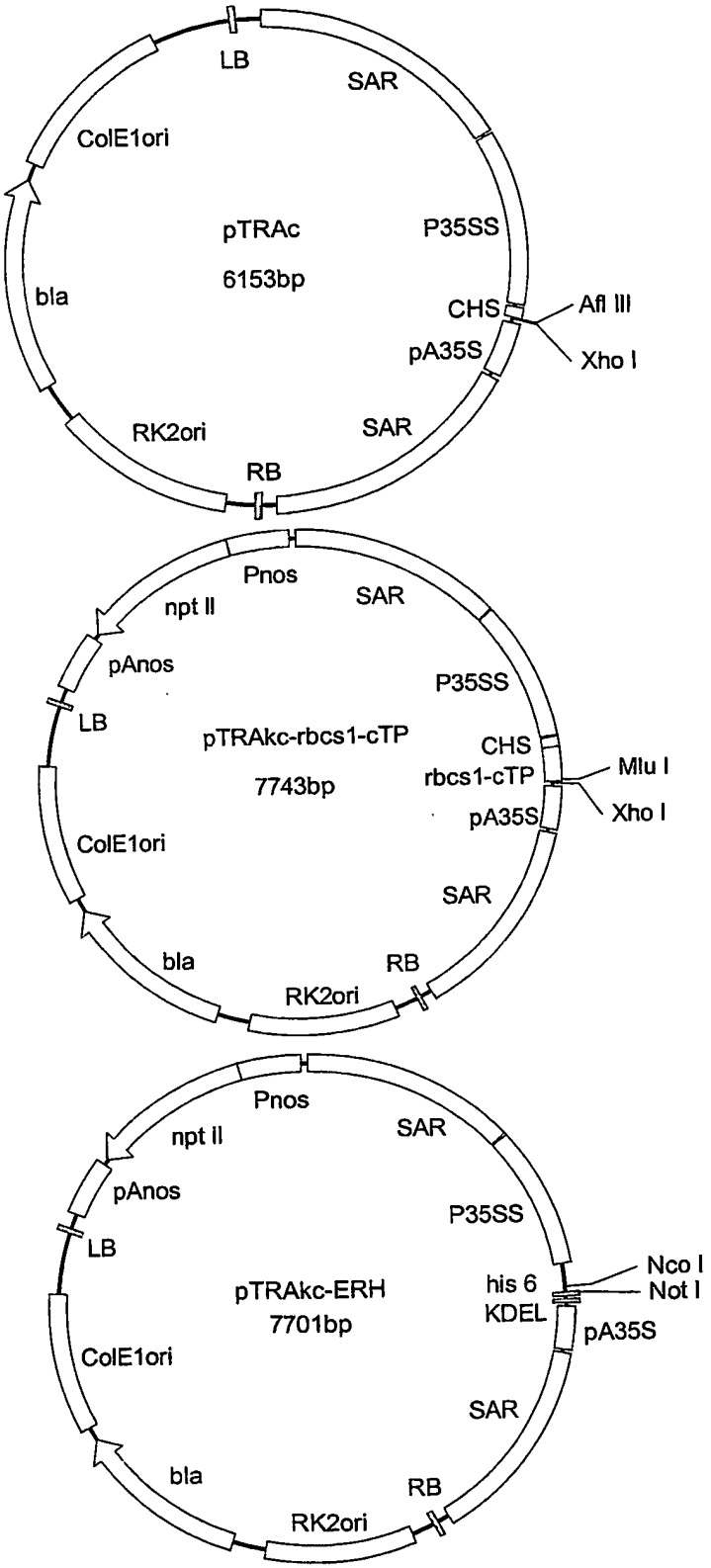

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SAR
bla
RB
RK2ori

Figure 1.

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Figure 2.

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Figure 3.

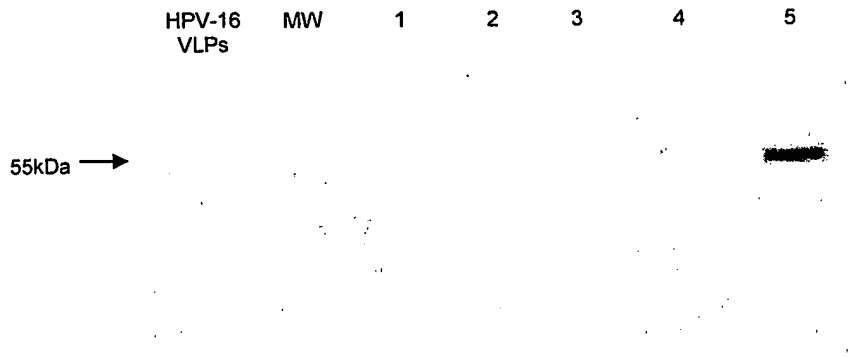


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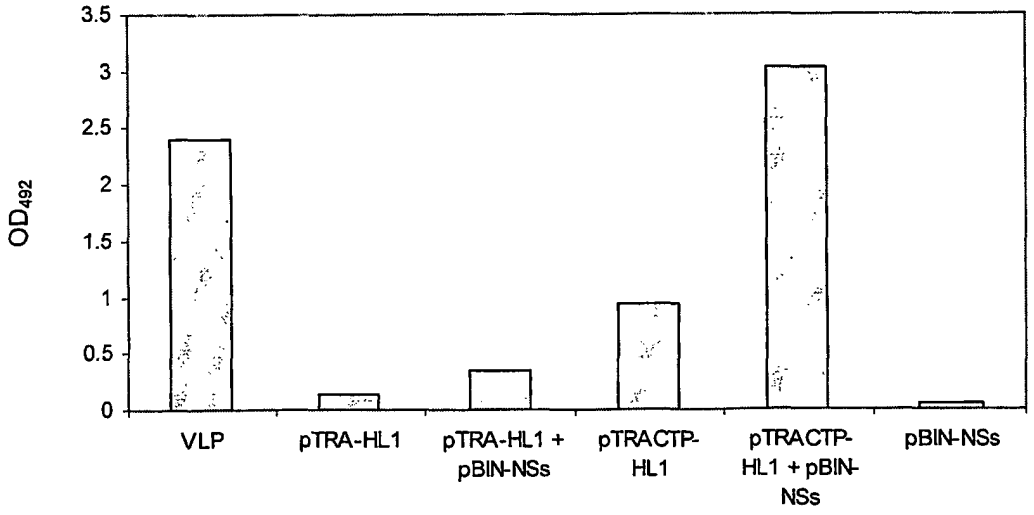


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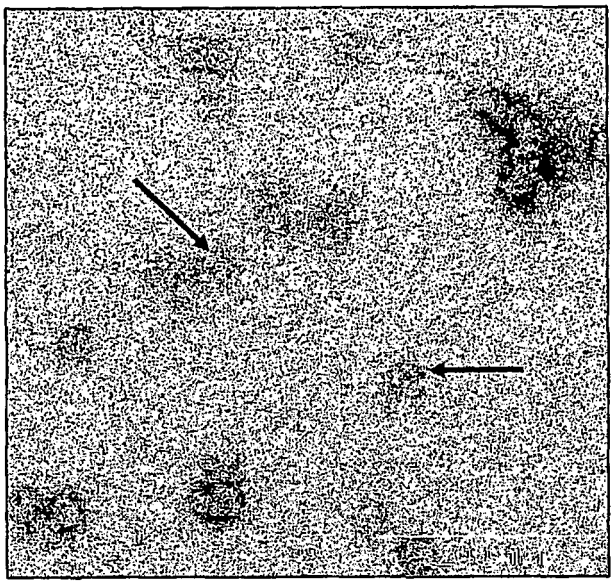


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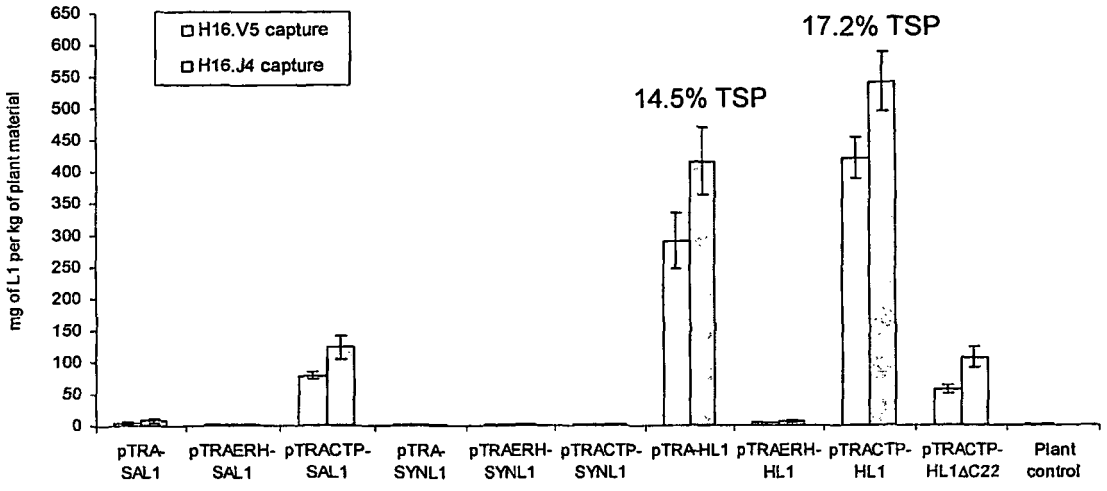


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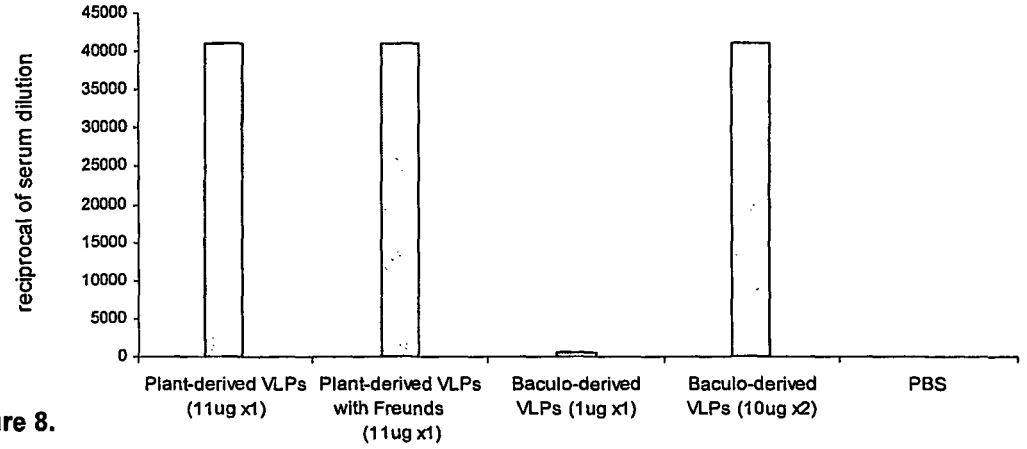


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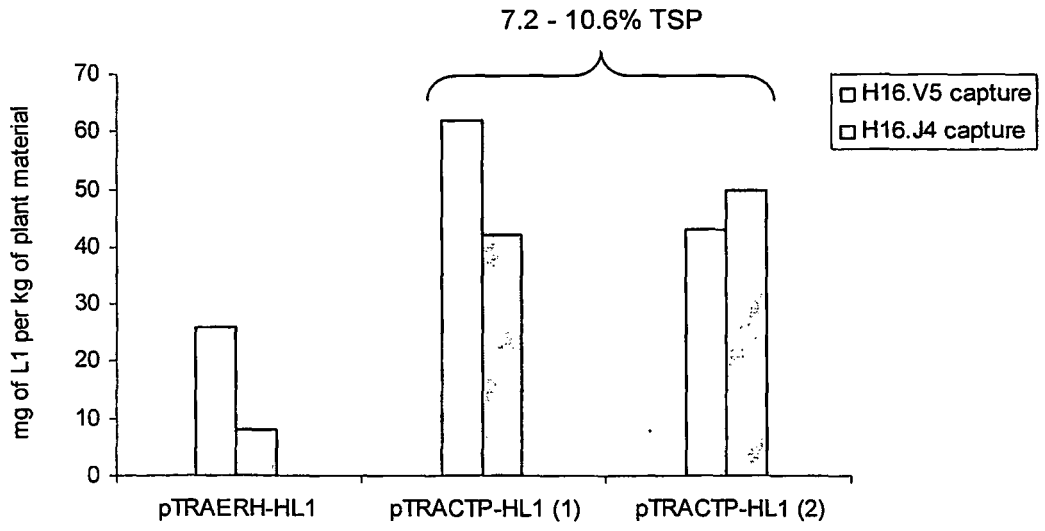


Figure 9.

H5

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Figure 10.

H5tr

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Figure 11.

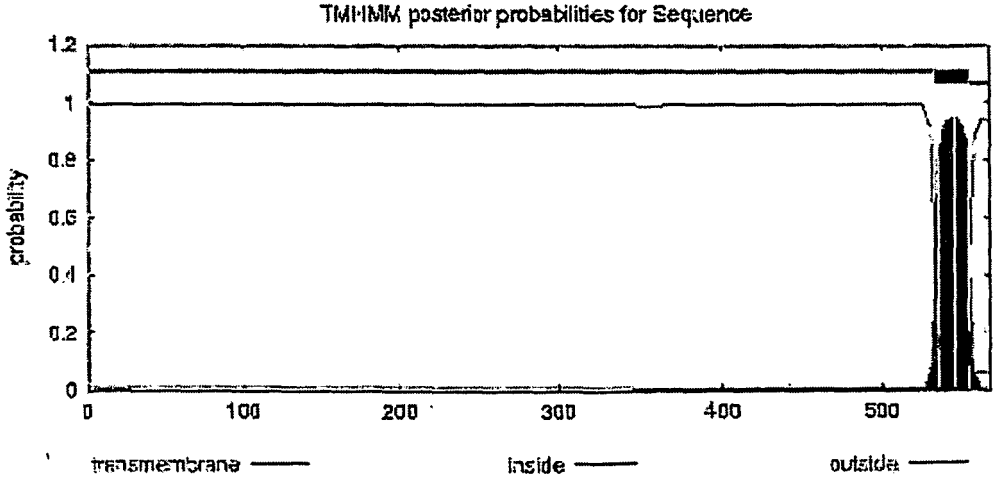


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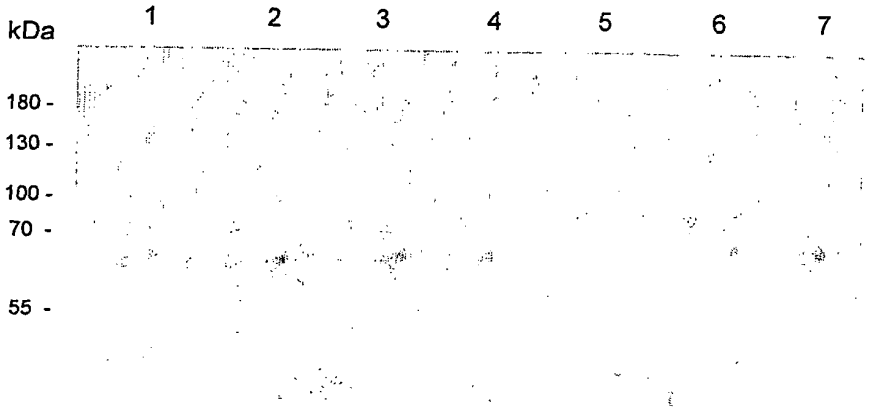


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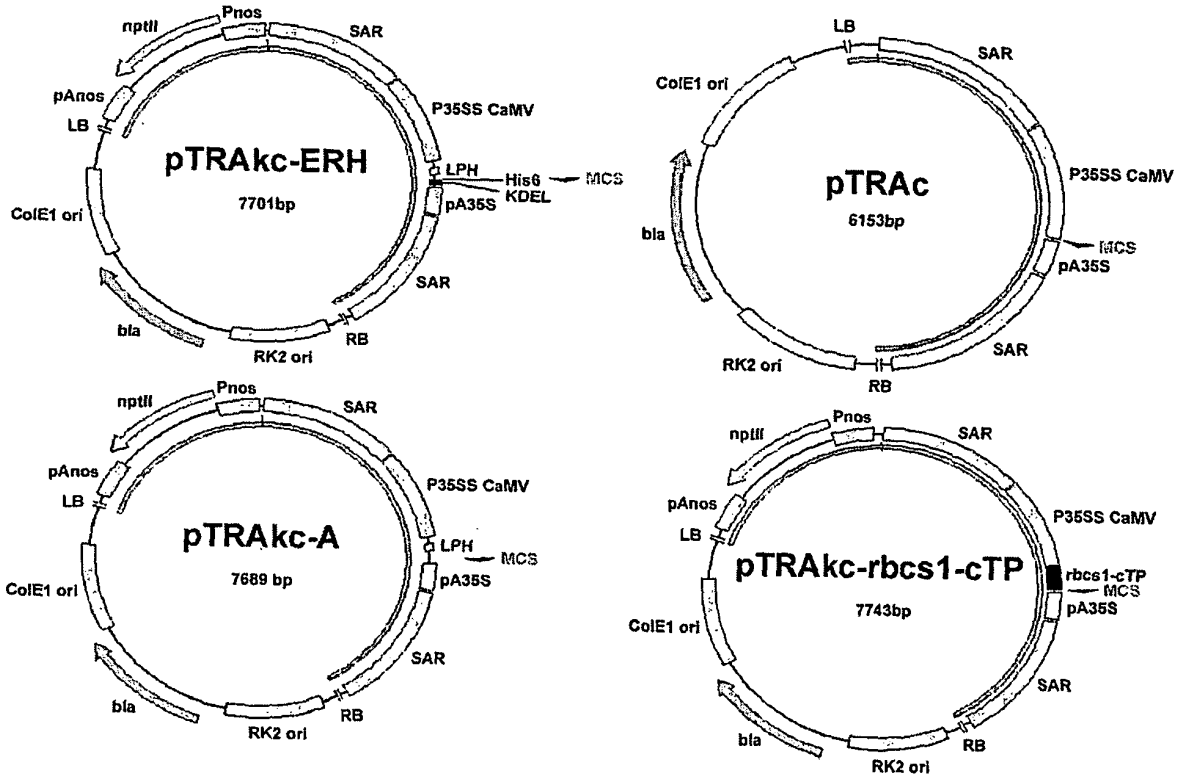


Figure 14

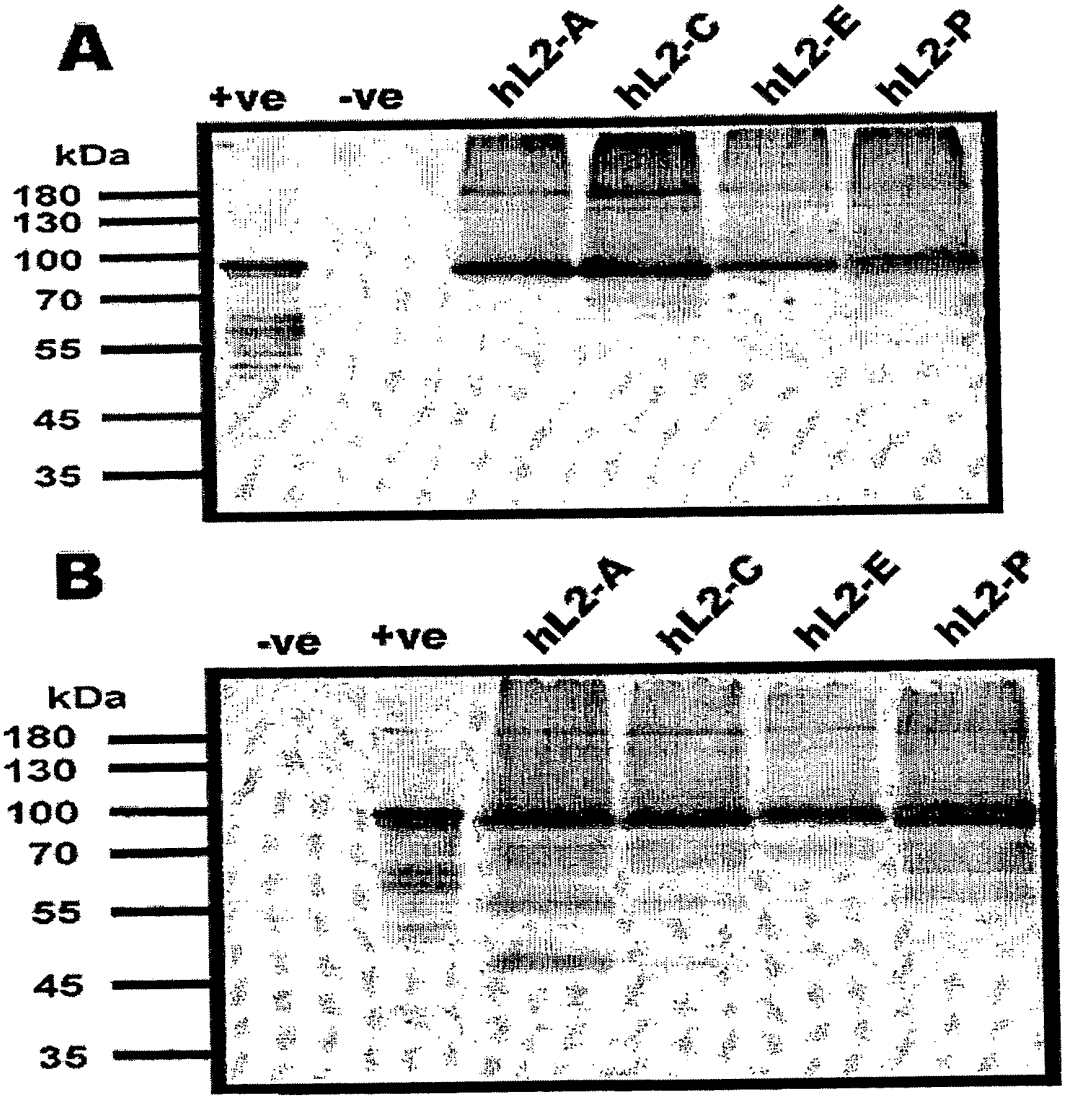

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Figure 15

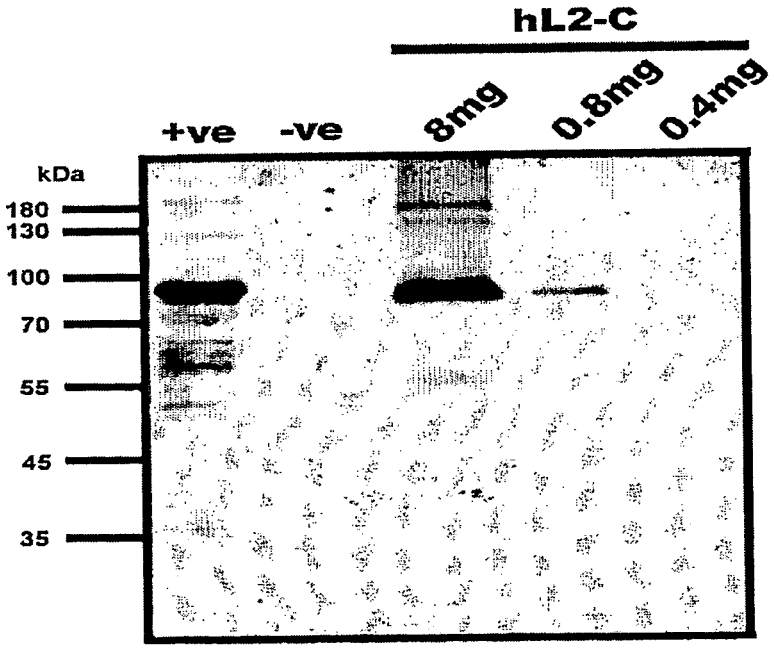


Figure 16

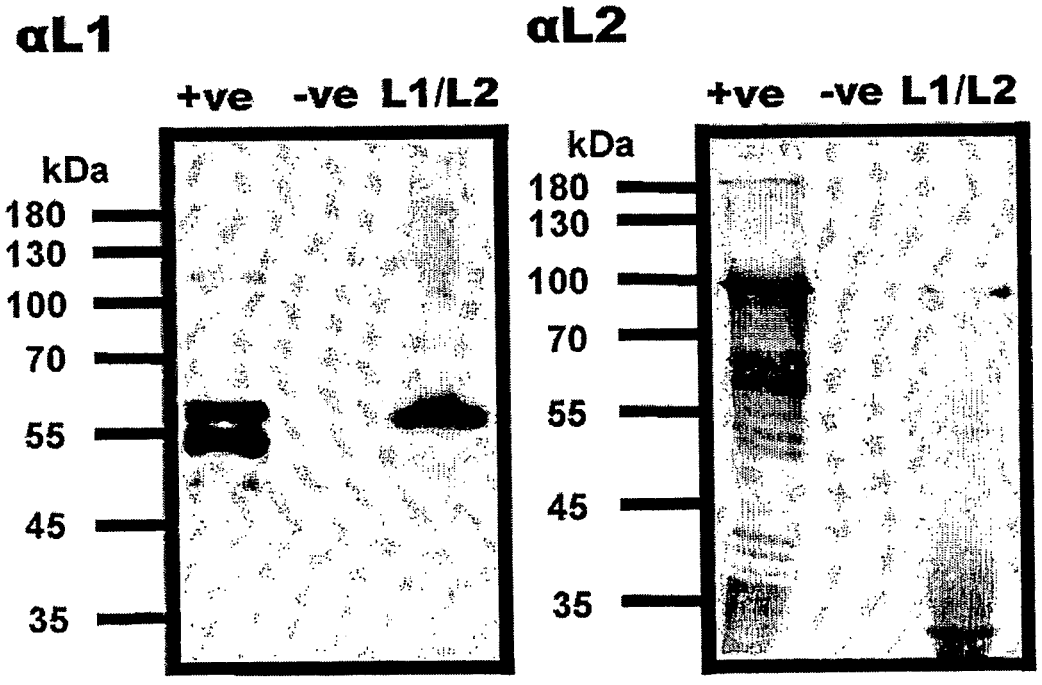


Figure 17

REFERENCES CITED IN THE DESCRIPTION

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